

**THE RESPONSE OF BREAST CANCER CELLS
TO *IN VITRO* SIMULATED HORMONE-
THERAPY AND IMMUNOTHERAPY**

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DECLARATION

I, Jacqueline Ferreira Gil (Student Number 0714760H), declare that this dissertation is my own. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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25th day of August, 2015.

ABSTRACT

Treatment of hormone-dependent breast tumours is typically conducted using hormone-therapy, with NK cell immunotherapy a novel modality. In this study, the effects of hormone-therapy and combined hormone-therapy with immunotherapy in MCF-7 breast cancer cells was investigated. A hormone pre-treatment of 17β -oestradiol and progesterone was performed to simulate the *in vivo* microenvironment. Subsequently cells were treated with the hormone-therapy drugs Anastrozole or RU486 alone, or with continued hormone simulation. Combined therapy was conducted with *in vitro* activated NK cells co-cultured with MCF-7 cells undergoing hormone-therapy.

Biomarkers ER α , PR and MUC1 were immunolocalised and expression analysed qualitatively, and quantitatively using image analysis software. Hormone pre-treatment reduced biomarker expression, stressing the importance of hormone environment simulation for *in vitro* experimentation. Hormone-therapy increased cytoplasmic ER α and decreased PR expression. Anastrozole increased MUC1 and RU486 decreased nuclear MUC1. With continued hormone simulation, Anastrozole further decreased all biomarkers whereas RU486 decreased ER α , increased PR expression with variable effects on MUC1 expression. RU486 induced MUC1/PR and MUC1/ER α correlation, which, under continued hormone simulation, was maintained in the nucleus only. Anastrozole induced MUC1/PR correlation in the cytoplasm which was maintained under continued hormone simulation. Hormone-therapy also induced a decrease in apoptosis, with continued hormone simulation abrogating Anastrozole induced apoptosis. While hormone-therapy did not increase proliferation, the associated changes observed in biomarker expression are linked with tumour progression indicating that short-term treatment may be detrimental for overall survival.

Combined NK cell immunotherapy resulted in decreased PR, while ER α and MUC1 expression increased in a hormone-dependent manner. Biomarker correlation was evident, albeit reduced with continued hormone simulation. Independently of hormone-therapy and hormone stimulation, immunotherapy reduced apoptosis, contrary to expectation. Proliferation was marginally reduced by immunotherapy.

The results indicate that immune cell function is inhibited by interaction with tumour cells, an effect that hormone-therapy cannot abrogate. Furthermore, that this study shows treatment

alters both nuclear and cytoplasmic expression of biomarkers, indicates that diagnostic procedures should consider both cellular compartments in tumour progression. It is further shown that qualitative analysis of biomarker expression is not always validated by quantitative analysis, with the latter proposed as a more objective and precise method to be used diagnostically.

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LIST OF ABBREVIATIONS

ACT Adoptive cell therapy	HCl Hydrochloric acid
ANAS Anastrozole	HT Hormone-therapy
ARB Antigen retrieval buffer	ICC Immunocytochemistry
BC Breast Cancer	IgG Immunoglobulin
BSA Bovine serum albumin	IL-2 Interleukin 2
CPT Camptothecin	IT Immunotherapy
CTL Cytotoxic T lymphocyte	MAPK Mitogen-activated protein kinases
DAPI 4', 6-diamidino-2-phenylindole	MB Miltenyi Buffer
DCC Dextran-coated charcoal	MCF-7 Michigan Cancer Foundation - 7
dDMSO DMSO diluent	MFI Mean fluorescent intensity
DMSO Dimethyl sulfoxide	MUC1 Mucin 1
dH₂O Distilled water	NK Natural Killer
DMEM Dulbecco's modified Eagle's medium	P/S Penicillin/Streptomycin
EDTA Ethylenediaminetetraacetic acid	PBMC Peripheral blood mononuclear cells
EP 17 β -oestradiol and progesterone	PBS Phosphate Buffered Saline
EPA 17 β -oestradiol, progesterone and Anastrozole	PHA Phytohaemagglutinin
EPR 17 β -oestradiol, progesterone and RU486	PI Propidium Iodide
ER Oestrogen receptor	PR Progesterone receptor
ERα Oestrogen receptor alpha	PRF Phenol-red free
ERE Oestrogen response element	PR Progesterone receptor
FBS Foetal bovine serum	PRE Progesterone response element
FITC Fluorescein isothiocyanate	Q Quadrant
FSC Forward scatter	RPMI Roswell Park Memorial Institute
	SSC Side scatter
	Tris Tris (hydroxymethyl)aminomethane

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INTRODUCTION

Breast cancer is the leading cancer amongst women worldwide, with the highest mortality rate (Jemal *et al.*, 2011; Torre *et al.*, 2015). In developing countries there is a lower incidence compared to developed countries (7.1% to 2.4%) but almost half of the diagnosed cases are fatal (1.2%). The increased incidence of breast cancer worldwide in the 1980s to 1990s has been linked to reproductive factors including oral contraceptive use, hormone replacement therapy and obesity (Jemal *et al.*, 2011; Torre *et al.*, 2015). However, in some developed countries the incidence rates have decreased in the last 25 years due to early detection and treatment (Torre *et al.*, 2015). In contrast, the rates in some developing countries have been increasing. While this is partially a factor of increased screening especially with mammography, the adoption of a more westernised lifestyle has been implicated in increasing breast cancer incidence (Jemal *et al.*, 2011; Youlden *et al.*, 2012).

Of particular concern in developing countries is access to treatment, with more targeted therapies not readily accessible for people of low socioeconomic status. Nevertheless, treatment for breast cancer, depending on subtypes, traditionally involves a mixed regimen of surgery, chemotherapy and radiation therapy, and hormone-therapy (Nicolini and Carpi, 2009). There have been many advances in the aforementioned treatment modalities, yet, even in developed countries the vast majority of patients survive only up to 5 years post-diagnosis (Youlden *et al.*, 2012). The low overall survival rate implicates recurrence and distant metastases (Youlden *et al.*, 2012). It is thus of paramount importance that attention is given to understanding alterations in breast cancer profiles under treatment. Furthermore, since the immune system has been shown to significantly influence tumour progression, more research is required in this regard.

Classification of Breast Tumours

Breast cancers are classified clinically according to their histopathology of which adenocarcinomas are the most prevalent; this is a tumour that originates from the epithelium and grows in a glandular pattern (Lester, 2015; Esebua, 2013). Adenocarcinomas are categorised by the anatomical location in the gland, whether ductal or lobular. These carcinomas are further classified by how far they have spread through the tissue: a carcinoma *in situ* has not spread beyond the basement membrane; however, an invasive/infiltrating

carcinoma has (Lester, 2015; Esebua, 2013). The majority of invasive breast tumours present as infiltrating ductal carcinoma (Lester, 2015; Esebua, 2013) .

Breast adenocarcinomas are further characterised by their molecular heterogeneity, which is linked with differing biological behaviour (Neve *et al.*, 2006) . Clinical treatment methods thus take into consideration a number of subtypes: Luminal, human epidermal growth factor receptor 2 (Her2)-enriched, Basal-like, normal breast-like and claudin-low (Eroles *et al.*, 2012; Miller, 2004). The Luminal phenotype of breast cancer is commonly referred to as hormone-dependent breast cancer and is characterised by the presence of the oestrogen receptor (ER) and progesterone receptor (PR) (Miller, 2004). The Luminal phenotype can be further subdivided into the Luminal A subtype which is ER⁺PR⁺HER2⁻, commonly found in post-menopausal patients (Eroles *et al.*, 2012; Miller, 2004); and the Luminal B subtype which is ER⁺PR⁺HER2⁻ with high Ki67 expression, a marker which indicates a high proliferative rate and associated with high metastatic potential (Eroles *et al.*, 2012). The Her2-enriched subtype is ER⁻PR⁻ but has an overexpression of HER2. The Basal-like subtype is conversely negative for the aforementioned markers (ER⁻PR⁻HER2⁻) and is commonly referred to as the triple negative phenotype. The Claudin-low subtype is similar to the Basal-like in that it is triple-negative; however, it also presents low expressions of proteins involved in intercellular adhesion (Eroles *et al.*, 2012).

These subtypes have been implicated in the differing of breast cancers in regards to their incidence, survival rate and treatment responses (Keller *et al.*, 2010; Prat and Perou, 2011; Parise and Caggiano, 2014). ER⁻ subtypes are more aggressive tumours and have been linked to increased mortality compared to the ER⁺ subtypes, with the ER⁺PR⁺HER2⁻ subtype (Luminal A and B) having the best survival rate (Parise and Caggiano, 2014). Thus, dependent on the subtype, treatments can be chosen accordingly (Eroles *et al.*, 2012).

In order to investigate breast tumour progression *in vitro*, established cell lines are commonly used. The MCF-7 cell line was established from a pleural effusion of a patient presenting breast adenocarcinoma (Neve *et al.*, 2006). Molecular studies indicate that MCF-7 cells are of a luminal phenotype (ER⁺ PR⁺); however, these cells show variance in the expression of HER2 (Neve *et al.*, 2006). Morphologically, in monolayer cell cultures, MCF-7 cells present a polygonal, ‘cobble-stone’ morphology, with distinct cell boundaries, typical of adenocarcinomas (Neve *et al.*, 2006). The MCF-7 cell line was used for this study.

Biomarker Expression in Luminal Breast Tumours

Of particular concern to this study is the luminal phenotype of breast adenocarcinoma, which as previously mentioned, is ER⁺PR⁺. Both ER and PR are classified as nuclear hormone receptors which mediate the effects of the steroid hormones, oestrogen and progesterone, respectively (Faivre *et al.*, 2005; Hill *et al.*, 2012; Renoir *et al.*, 2013). ER α is overexpressed in ER⁺ breast tumours and modulates their response to oestradiol (a derivative of oestrogen), particularly with regard to cell proliferation and cell survival (Renoir *et al.*, 2013). PR is also overexpressed in PR⁺ breast tumours and interacts with progestins (a derivative of progesterone) (Faivre *et al.*, 2005). To better understand the role of these steroid hormones in tumourigenesis reference can be made to their original role in mammary development. Specifically, 17 β -oestradiol mediates outgrowth and branching of ducts, whereas progesterone mediates branching and the development of the secretory epithelium, for subsequent lactation. This action continues through the menstrual cycle (Gellersen *et al.*, 2009; Knutson and Lange, 2014). Lifetime exposure and high levels of oestrogen and progesterone, are associated with increased risk for breast cancer, particularly the luminal phenotype which is well known for its hormone-dependency in tumour progression, specifically in initiation, cell proliferation and survival mechanisms (Mendelson and Hardy, 2006; Knutson and Lange, 2014).

There are two different isoforms of ER: ER α and ER β ; with three different isoforms of the PR noted: PR-A, PR-B and PR-C. The isoforms of both the ER and PR share a similar structure with an N-terminal domain (NTD), a DNA binding domain (DBD), a hinge domain and a C-terminal domain (CTD) (Acconcia and Kumar, 2006; Lange, 2008; Hill *et al.*, 2012). The NTD allows for intermolecular and intramolecular associations as well as gene transcription activation (Acconcia and Kumar, 2006; Hill *et al.*, 2012). The DBD binds the receptor to hormone response elements (HRE) in the promoter regions of targeted genes (e.g. *c-myc*), i.e. ER binds to oestrogen response elements (EREs) and PR binds to progesterone response elements (PREs) (Acconcia and Kumar, 2006; Lange, 2008; Hill *et al.*, 2012). The hinge domain is located between the DBD and the CTD and when the receptor is unliganded, allows binding to chaperone heat shock proteins (Hsp) to modulate translocation from the cytoplasm to the nucleus (Acconcia and Kumar, 2006; Hill *et al.*, 2012). The CTD contains the ligand binding domain (LBD) and the F domain. The LBD, as its name suggests, is the location at which the hormone is bound; however, it also interacts with the DBD to modulate

gene transcription. The F domain also functions to modulate transcriptional activity (Acconcia and Kumar, 2006; Hill *et al.*, 2012). In contrast to the classical PR-A and PR-B, PR-C has a truncated DBD which doesn't allow for activation of transcription, however, it does increase the transcriptional activity of the classical PRs (Wei *et al.*, 1997).

The actions of these hormone receptors differ depending on their cellular location, mainly cytoplasmic and nuclear location (Acconcia and Kumar, 2006; Lange, 2008; Hill *et al.*, 2012). Interestingly, it has been discovered that ER and PR can function independently of the classical genomic pathway (Acconcia and Kumar, 2006; Gellerson *et al.*, 2009). The genomic pathway states that unliganded, the receptors are bound to Hsp and inactive, and when bound to a ligand dissociate from Hsp and translocate to the nucleus to bind to HREs. However, these receptors also function via a non-genomic pathway, where sequestration in the cytoplasm allows these hormones to act without the use of transcription and instead interact with other receptors or cytoplasmic kinases (e.g.: Src) (Acconcia and Kumar, 2006; Lange, 2008; Hill *et al.*, 2012; Welsh *et al.*, 2012). It is proposed that these two pathways are not independent of each other but act in concert to provide a response to the hormones (Acconcia and Kumar, 2006; Lange, 2008; Hill *et al.*, 2012), while other studies note that nuclear and cytoplasmic ER may function in direct conflict – promoting or inhibiting tumour progression (Björnström and Sjöberg, 2004).

The localisation of ER and PR in both the nuclear and cytoplasmic compartments are thus of paramount concern. Clinically, tumour immunohistochemical assessment of ER is limited to the nucleus and cytoplasmic expression is not considered (Welsh *et al.*, 2012). Given that biologically, ER and PR function may not be dependent on purely the genomic pathway, cytoplasmic assessment of hormone receptors could be of diagnostic and prognostic significance, as well as an indication for hormone-therapy types (Welsh *et al.*, 2012).

A new biomarker that has also been implicated in breast tumour progression is mucin-1 (MUC1), a large multi-unit transmembrane glycoprotein. This glycoprotein is normally expressed on the apical surface of secretory epithelial cells where it plays a protective role as part of the glycocalyx (Wei *et al.*, 2006). MUC1 has two subunits, the N-terminal (MUC1-N) and C-terminal (MUC1-C) subunit. The MUC1-N subunit is large, extending beyond the glycocalyx, with the MUC1-C subunit tethering the MUC1-N subunit to the cytoskeleton (Wei *et al.*, 2006). During tumourigenesis glycosylation patterns change and MUC1 becomes homogenously and overexpressed, both in the cell and the tumour as a whole. This has been

specifically noted in breast tumours and thus MUC1 has been established as an oncoprotein (Wei *et al.*, 2006; Nath and Mukherjee, 2014).

In tumour progression MUC1 mediates the secretion of a number of growth factors, inducing pro-survival and proliferation mechanisms (Kufe, 2009; Nath and Mukherjee, 2014). Moreover, MUC1 is implicated in the induction and maintenance of epithelial-mesenchymal transitions, via nuclear translocation of the cytoplasmic domain of MUC1-C where its action results in the upregulation of transcription factors *Snail*, *Slug*, *Vimentin* and *Twist*, known inducers of invasive phenotypes (Nath and Mukherjee, 2014). MUC1 functions in the inhibition of apoptotic mechanisms via the downregulation of pro-apoptotic and upregulation of anti-apoptotic members of the Bcl-2 family (Nath and Mukherjee, 2014). Furthermore, MUC1 interacts directly with ER- α stimulating co-activator binding as well as ER α -mediated transcription (Wei *et al.*, 2006). There are currently no reports of the interaction between PR and MUC1 in breast cancer. However, in human uterine cells PR acts differentially, with liganded PR-B stimulating and PR-A inhibiting MUC1 expression (Brayman *et al.*, 2006). Clinically, breast tumour cytoplasmic MUC1 expression has been shown to be significantly associated with PR expression (van der Vegt *et al.*, 2007).

In metastasis, MUC1 is proposed to prevent cell-cell adhesion due to the large extension and aberrant glycosylation of its N-terminal subunit thus promoting cell migration (Gendler, 2001). An increase in MUC1 in K562 cells has been shown to inhibit Natural Killer (NK) cell mediated lysis *in vitro* (Zhang *et al.*, 1997). Also the secretion of MUC1 is associated with immune evasion processes including the inhibition of lymphocyte migration to the tumour site and inhibition of NK cell function (Gendler, 2001; Suzuki *et al.*, 2012; Villalba *et al.*, 2013). The increased MUC1 expression in many epithelial cancers is introducing a number of MUC1 targeted therapies which are currently under investigation (Mukhopadhyay *et al.*, 2011; Nath and Mukherjee, 2014). MUC1 has been further shown to be expressed in the luminal phenotype MCF-7 cell line (Gil *et al.*, 2013), which made this cell line ideal for this study.

Analysis of Biomarker Expression in Breast Cancer

The most cost-effective method to classify breast tumours is visually via immunohistochemical assays which can assess receptor expression (e.g. ER, PR, HER2), oncogene presentation (e.g. *myc*) and proliferation markers (e.g. Ki67) (Shyyan *et al.*, 2006). However, such assessment requires a qualified, experienced pathologist to interpret the results. Lack of experience or capacity as evident in many developing countries, implicates subjectivity and possible false negatives or positives in such circumstances (Anderson *et al.*, 2008). Furthermore, as previously mentioned, the biomarkers ER, PR and MUC1, which are of considerable importance to defining breast cancer subtype, and thus eliciting a treatment strategy, are only considered when localised to the nucleus (Welsh *et al.*, 2012). A new strategy is required to combine the plethora of data indicating cytoplasmic function in tumour progression with new tools for diagnostic analysis.

A new technique to study these assays would be objective quantification of the images produced, which could be performed using image analysis software (Rexhepaj *et al.*, 2008; Burguera *et al.*, 2010). We have previously used the online, free image analysis software ‘CellProfiler’ (Carpenter *et al.*, 2006) to analyse the expression of MUC1 and ER α with immunohistochemistry (Gil *et al.*, 2013). For this study, such software allows for identification of stained components within cells. This allows for assessment of staining intensity and localisation to cellular compartments, amongst numerous other functions including determination of cell number, cellular compartment number and size measures (Carpenter *et al.*, 2006). Sophisticated algorithms allow for the generation of ‘pipelines’ – step-by-step procedures – which allow for high imaging throughput (Carpenter *et al.*, 2006). The same parameters would be applied to each image, thus subjectivity, as in qualitative assessment, would not be a defining factor of any study. Furthermore, once data is generated, sophisticated statistical techniques may be employed to find subtle relationships that would otherwise be hidden by qualitative analysis alone (Gil *et al.*, 2013).

Hormone-Therapy of Hormone-Dependent Breast Cancer

Hormone therapy for hormone-dependent tumours has evolved from oophorectomy, where in 1896, Beatson showed a regression in metastatic breast cancer in two women who had undergone this surgery (Beatson, 1896; Dao, 1972; Enmark and Gustafsson, 1999; Speirs *et al.*, 2004). The oestrone hormone was later isolated in 1929 and led to the discovery of a relationship between this hormone and breast cancer. The concept of hormone-dependence was realised in the 1940s with Huggins' experiments of orchiectomy with regards to prostatic cancer. Jensen discovered the oestrogen receptor in 1958 (Dao, 1972; Enmark and Gustafsson, 1999; Speirs *et al.*, 2004) and in 1967 it was proposed that breast cancers may also contain hormone receptors like their non-cancerous counterpart and would therefore be responsive to hormone manipulation (Edwards *et al.*, 1979). This discovery instigated the use of procedures to restrict hormones in both prostate cancers and breast cancers and to discover the mechanism by which this worked (Dao, 1972). In 1974, it was determined that 60% of breast cancers that contained ER were susceptible to oestrogen manipulation (Edwards *et al.*, 1979). The fact that not all carcinomas containing ER were susceptible to endocrine therapy led to the theory that progesterone and the progesterone receptor were also involved (Edwards *et al.*, 1979). This allowed for novel experimentation in therapeutic intervention strategies that were directed at these receptors.

Chemical oophorectomy, in which luteinizing hormone-releasing hormone (LH-RH) or gonadotropin-releasing hormone (GnRH) analogues are used to induce ovarian suppression has been indicated for premenopausal women with ER⁺ breast cancer (Ray and Ficek, 2012). However, it was found that most ER⁺ breast tumours present in post-menopausal women and these patients produce oestrogen extra-gonadally via the aromatase enzyme (Ray and Ficek, 2012). The local biosynthesis of oestrogen appears associated with developing a more aggressive disease, a poorer outcome as well as increased local and distant recurrences (Kulendran *et al.*, 2009). Thus in most cases chemical oophorectomy is not commonly used as a treatment modality, but rather the current hormone-therapies including selective oestrogen receptor modulators (SERM), selective oestrogen receptor downregulators (SERD), and aromatase inhibitors (AI) (Howell *et al.*, 2004; Ray and Ficek, 2012). These therapies are usually used as an adjuvant (post-surgery) or neo-adjuvant (pre-surgery) treatment.

SERMs function as receptor antagonists primarily; however, they also function as agonists on non-breast tissues (e.g. endometrium and bone) (Jordan, 2001). The first SERM generated was Tamoxifen, which was discovered to inhibit induced mammary carcinogenesis in rats in 1975, followed by widespread clinical use (Jordan, 2001, 2008). Since then, this drug has been the standard adjuvant hormone-therapy for both pre-menopausal and postmenopausal women (Howell *et al.*, 2004; Ray and Ficek, 2012). SERDs are classified as pure oestrogen antagonists; however, the only SERD in use clinically is fulvestrant which is used as a third line treatment (Howell *et al.*, 2004; Ray and Ficek, 2012), particularly after patients develop resistance to Tamoxifen treatment (Knutson and Radisky, 2013).

When it was discovered that oestrogen is produced in breast carcinomas themselves, independently of the ovaries (Miller and Forrest, 1974) and that the level was significantly higher than normal breast tissue (van Landeghem *et al.*, 1985), the use of AIs to inhibit the accumulation of oestrogen in breast carcinomas was investigated. AIs bind to the cytochrome P450 aromatase enzyme which prevents it from converting androgens to oestrogens (Miller, 2004). The first AI to be developed was the non-steroidal aminoglutethimide in 1974 and was dubbed a first generation AI; however, this AI was not specific to aromatase but also inhibited other cytochrome P450 enzymes (Brueggemeier *et al.*, 2005). The next AI developed was formestane, a second generation AI, and although it was more effective and more aromatase specific than aminoglutethimide, it still had some non-specific binding (Brueggemeier *et al.*, 2005). The last jump in AI development was the third generation AIs which did not affect other cytochrome P450 enzymes; these include the non-steroidal vorozole, letrozole, anastrozole AIs and the steroidal exemestane AI (Miller, 2004; Brueggemeier *et al.*, 2005; Kulendran *et al.*, 2009). Anastrozole, the most widely used of these AIs producing the greatest decrease in oestrogen levels (Brueggemeier *et al.*, 2005; Kulendran *et al.*, 2009), is of particular interest to this study.

Research has shown that in postmenopausal women third generation AIs are superior to Tamoxifen with regards to inhibition of breast cancer progression and are currently used as a first line treatment for this demographic (Kulendran *et al.*, 2009; Ray and Ficek, 2012). In patients treated with Anastrozole or Letrozole for 14 days before surgery, ER and PR expression, as well as tumour cell proliferation was significantly reduced (Murray *et al.*, 2009). Anastrozole 15 week treatment also decreased endogenous oestrogen levels in breast tumours (Geisler, 2003). The phase III trial ATAC (Anastrozole, Tamoxifen Alone, or in

Combination) showed that Anastrozole significantly increased disease-free survival, time-to-reoccurrence and decreased distant metastases in postmenopausal women with early hormone-dependent breast cancer (ATAC Trialists Group, 2005). In the IBIS-II (International Breast Cancer Intervention Study II) trial, prophylactic Anastrozole decreased the incidence of breast cancer in postmenopausal women who were at an increased risk of breast cancer (Cuzick *et al.*, 2014).

While these results indicate that reduction of hormone receptors may be an ideal expectation, clinically downregulation of ER α expression is associated with increased recurrence and metastasis (Dhasarathy *et al.*, 2007). Moreover, with prolonged treatment ER⁺ breast tumours have been shown to acquire resistance, which is still not fully understood but is known to include increased ER co-regulators and receptor cross-talk (Renoir *et al.*, 2013). Furthermore, during the metastatic process, circulating tumour cells derived from ER⁺ tumours have been shown to downregulate their ER profile (Aktas *et al.*, 2011), thus rendering ER hormone-therapy inadequate.

It is now well-known that oestradiol promotes the progression of hormone-dependent tumours. However, over the years there have been widely differing opinions on whether progesterone has a proliferative and/or metastatic effect on breast cancer. Early studies indicated that progesterone treatment inhibited tumourigenicity of pre-cancerous cells but promoted tumourigenicity of cancerous cells (Horwitz, 1992). It was further proposed that progesterone inhibits breast cancer proliferation (Parazzini *et al.*, 1993). Conversely, more recent studies indicate that progesterone alone or in combination with 17 β -oestradiol increases the proliferative and metastatic potential of breast cancer cells (Faivre *et al.*, 2005; Fu *et al.*, 2008). Clinical data shows that high levels of progesterone are associated with increased risk for breast cancer, with other studies indicating that progestins are implicated in tumour cell cycle progression (Faivre *et al.*, 2005; Knutson and Lange, 2014).

Despite the contention around progesterone's effects on cancer, a new type of hormone-therapy is being researched, that of anti-progestins, or PR antagonists. The most common anti-progestin undergoing study is RU486, also known as mifepristone or the morning-after pill, and of particular interest to this study. This drug has a greater affinity for PR compared to progesterone (Vegeto *et al.*, 1992), thus it binds to the PR, but at a more N-terminal location than that of progesterone (Vegeto *et al.*, 1992). However, it has been identified that RU486 has weak oestrogenic activity due to its steroid nature, binding to the hormone-binding domain of

the ER (Jeng *et al.*, 1993). RU486 can also act as a PR agonist under certain conditions mainly with PR-B undergoing conformations in the NTD (Hill *et al.*, 2012). It has been determined that the inhibitory effect of RU486 on cancer cells is not dependent on nuclear PR expression (Tieszen *et al.*, 2011; Chen *et al.*, 2014); however, the mechanism of PR-independent RU486 action remains unknown.

RU486 has been shown to significantly inhibit tumour growth as well as have various other anti-tumour effects in 24 cell lines, including MCF-7 (Chen *et al.*, 2014). With regard to induced tumours in rats, RU486 treatment was able to inhibit tumour growth (Bakker *et al.*, 1987). The first clinical trial was with 200 mg/day RU486 as a treatment for metastatic breast cancer in postmenopausal women, this induced partial regression or stabilisation of the tumour in over half the patients (12 out of 22) (Romieu *et al.*, 1987). Another trial at 200-400 mg/day also showed stabilisation in 6 patients (out of 11) (Klijn *et al.*, 1989). However, in 1996, a trial with postmenopausal patients with PR⁺ metastatic breast cancer treated with RU486 showed a low overall response rate (Perrault *et al.*, 1996). RU486 has been suggested to have a protective effect on normal breast epithelium (Engman *et al.*, 2008). Clinical trials conducted from 2012 - 2014 tested this theory, by treating healthy patients presenting with the BRCA1/2 mutation and thus regarded as a high risk group for breast cancer, with RU486 (Danielsson, 2015). The results have yet to be published.

Interestingly, an association between ER and PR has been discovered and this has implications for treatments which target only the one receptor. There has been identified a ligand-independent direct association of the ER with PR-B, with regard to the c-Src/p21ras/Erk-2 transducing pathway (Migliaccio *et al.*, 1998). The C-terminal domain of ER binds to the N-terminal domain of PR-B, which is absent in PR-A. This permits progesterone to stimulate the binding of the ligand-free ER to the proto-oncogene c-Src and activate the subsequent cascades leading to tumour progression. Conversely, the stimulation of this pathway by oestrogen is independent of PR-B (Migliaccio *et al.*, 1998), indicating a role for progesterone in PR^{-/low} breast tumours .

Given these findings, a combination of hormone-therapy treatments to target both oestrogen and progesterone has been proposed and investigated *in vitro*. Simultaneous treatment of MCF-7 cells with RU486 and Tamoxifen induced a synergistic inhibitory effect on the proliferative rate for short treatment (< 3 days) (Thomas and Monet, 1992) and significantly increased cell growth inhibition, DNA fragmentation, bcl₂ downregulation, induction of transforming growth

factor β_1 (TGF β_1), as well as translocation of protein kinase C activity (El Etreby *et al.*, 1998), all of which contribute to tumour progression. In induced mammary tumours in rats, a combination of RU486 and Tamoxifen induced a greater tumour regression compared to each alone (Bakker *et al.*, 1990). However, the efficacy of this combination therapy has yet to be confirmed by additional clinical studies.

Further interactions have been noted between ER, PR and MUC1. In patients with primary ductal carcinoma apical membrane i.e. non-transformed MUC1 expression has been linked to smaller tumours, lower tumour grades, PR positivity and increased overall survival (van der Vegt *et al.*, 2007). Diffuse cytoplasmic MUC1 expression has been linked to cyclin D1 positivity and increased relapse-free survival. However, MUC1 has also been determined to be an independent risk factor for poor relapse free survival and overall survival (van der Vegt *et al.*, 2007). Under oestradiol stimulation, MUC1 has been implicated in interacting directly with the ER α and thus stimulating co-activator binding as well as ER α -mediated transcription (Wei, *et al.*, 2006), which in breast tumours, is implicated in promoting tumour progression. With patients undergoing 3 weeks Tamoxifen treatment before surgery, it was determined that a lower percentage of tumour cells expressed MUC1 and that this expression was lower (Hanson *et al.*, 2001), corresponding to a possible ER-mediated regulation of MUC1. In our laboratory, we have further shown that in the absence of exogenous hormones, short-term treatment (3 days) with Anastrozole and RU486 increase both ER α and MUC1 in MCF-7 cells (Gil *et al.*, 2013).

Furthermore, since MUC1 is implicated in facilitating adhesion, many studies have assessed the relationship between the PR and MUC1 linked to embryo implantation. Studies conducted on endometrial cell lines indicate that PR regulates MUC1 gene expression, with progesterone itself capable of upregulating MUC1 expression (Horne *et al.*, 2006; Dharmaraj *et al.*, 2010). Since both the endometrium and breast tissue are hormonally regulated, we postulate that in luminal phenotype tumours, MUC1 function may be linked with PR or progesterone directly.

Immunotherapy of Breast Cancer

The immune system can recognize and destroy cancerous cells; however, many cancers employ immune evasion strategies that circumvent the immune system (Lechner *et al.*, 2011). The immune system is made up of the innate immune system and the adaptive immune system. The innate immune system comprises of non-specific defence mechanisms using macrophages and neutrophils, while the adaptive immune system comprises of antigen-specific defence mechanisms mediated by B cells and T cells (Janeway *et al.*, 2001). Lymphocytes originate from stem cells in the bone marrow; the B-cells undergoing antigen-independent development in the bone marrow and T-cells, in the thymus (Janeway *et al.*, 2001). T cells are divided into helper T (T_H) cells ($CD4^+$) and cytotoxic T lymphocytes/cells (CTLs or T_C cells) ($CD8^+$). $CD4^+$ T_H cells recognise class I histocompatibility molecules (HLA-A, HLA-B, and HLA-C) and $CD8^+$ CTLs recognise class II histocompatibility molecules (HLA-D) (Roth *et al.*, 2006). Another type of lymphocyte involved in cytotoxicity is NK cells, of particular interest to this study. NK cells detect cellular surface changes; however, these cells are regarded as part of the innate immune system and use a different mechanism to T-cells for antigen recognition (Roth *et al.*, 2006).

NK cells differentiate between cancerous cells and healthy cells by detecting the balance between activating and inhibitory signals, although inhibitory signals can overrule activating signals as a safeguard mechanism (Yawata *et al.*, 2008; Blattman and Greenberg, 2004). The activating receptors on NK cells include NKG2D, NKp30, NKp46, DNAM-1, CD2, NKp80, 2B4, and NTBA; while the inhibitory receptors include NKG2A, CD85j, and KIR. NK cells also express CD16 which is linked to the induction of antibody-dependent cellular cytotoxicity. NK cell activation leads to NK-cell induced apoptosis, NK cell proliferation and cytokine release (Yawata *et al.*, 2008; Blattman and Greenberg, 2004). NK cells are traditionally classified as $CD3^-CD56^+$ lymphocytes forming part of the innate immune response, whereby they induce apoptosis in their targeted cells. Two NK cells populations have been ascertained depending on $CD56^+$ expression, they are known as $CD56^{dim}$ and $CD56^{bright}$ (Frag and Caligiuri, 2006).

Tumours do not exist in isolation with only the neoplastic cells; but in what has been termed the tumour microenvironment (TME) containing a wide range of cell types including fibroblasts, endothelial cells and cells of the immune system (Leibovici *et al.*, 2011). The TME also includes the extracellular matrix as well as diverse soluble molecules. The immune cells

contained within the TME are variable and in most cancers the tumours are able to evade the immune system and promote tumour progression. The TME contains immune cells of both the innate and adaptive immune system including macrophages, dendritic cells, basophils, mast cells, eosinophils, NK cells and T cells (Witz, 2009; Leibovici *et al.*, 2011). Regulatory immune cells are also found in the TME, including T regulatory (Tregs) cells and myeloid-derived suppressor cells (MDSCs) (Stewart and Abrams, 2008; Leibovici *et al.*, 2011).

In breast cancers, it has been found that the macrophages and Tregs found in the TME promote tumour progression. Macrophages are implicated in angiogenesis and ECM generation while the Tregs suppress T cell, B cell and NK cell function. Macrophage infiltration or increased Treg infiltration in breast tumours denotes poor survival (Muranski *et al.*, 2006; Mougiakakos *et al.*, 2010). A role for CD8⁺ T cells in breast cancer has not been fully elucidated; the data available is conflicting indicating that further research is necessary to understand CD8⁺ T cells in breast cancer (Knutson and Radisky, 2013). Interestingly, the breast TME is lacking in NK cells (Georgiannos *et al.*, 2003; Macchetti *et al.*, 2006), despite elevated peripheral blood NK cell levels (Mozaffari *et al.*, 2007), which may indicate evasion of the immune system (Leibovici *et al.*, 2011; Albertsson *et al.*, 2003). However, in cancers in which NK cells have invaded the tumour infiltrate there is an association with a better prognosis. These cancers include squamous-cell lung carcinoma, gastric carcinoma and colorectal carcinoma (Albertsson *et al.*, 2003). This improved infiltration has been linked to NK cell activation and thus NK cell function (Albertsson *et al.*, 2003).

Given the importance of the immune system in tumour progression, a branch of treatment - immunotherapy is being investigated (Gattinoni *et al.*, 2006). This treatment manipulates the immune system to treat cancers. The various subtypes of immunotherapy to date include non-specific immunomodulation (NSI), monoclonal antibody (mAB) therapy, cancer vaccines and adoptive cell transfer (ACT) (Gattinoni *et al.*, 2006; Rosenberg *et al.*, 2008). Non-specific immunomodulation is the use of immunomodulators to activate endogenous immune cells and currently the only modulator in use is interleukin-2 (IL-2), a potent immunostimulatory cytokine; however, others are under investigation including IL-15, anti-transforming growth factor- β (anti-TGF β) and anti-programmed death 1 (anti-PD-1) (Rosenberg *et al.*, 2008). mAB therapy involves targeting tumour associated antigens and is thus very much dependent on the discovery of biomarkers that delineate cancerous tissue from normal tissue. Cancer vaccines consist of the immunisation of a patient via whole cells, proteins or other vectors which has had

very little success to date (Gattinoni *et al.*, 2006; Rosenberg *et al.*, 2008; Leibovici *et al.*, 2011; Dougan and Dranoff, 2012).

Of particular concern to this study is ACT. ACT is the identification of anti-tumour lymphocytes *ex vivo*, which are then activated and expanded *in vitro* and then transferred into cancer patients (Gattinoni *et al.*, 2006; Rosenberg *et al.*, 2008; Geller *et al.*, 2011). These lymphocytes can either be obtained from circulation or from populations known as tumour infiltrating lymphocytes (TIL). ACT has been performed with TILs expanded with IL-2 *in vitro* and used to successfully treat lung and liver tumours in mice (Rosenberg *et al.*, 1986, 2008). Clinically, successful tumour regression of melanomas was achieved using TILs isolated from melanomas and expanded with IL-2 transferred post-lymphodepletion (Rosenberg *et al.*, 1988; Rosenberg *et al.*, 2008). ACT has been primarily conducted using T cells due to the fact that they are part of the adaptive immune system and can be targeted to tumour-specific antigens (June, 2007). However, in cancers in which NK cells have invaded the tumour, there is an association with a better prognosis (Albertsson *et al.*, 2003).

It has been revealed that breast cancer cells modify their MHC receptors to escape from NK cell activity, undergo immuno-editing to change their mRNA and protein expression to NK cells, produce a suppressive environment and prevent NK cell maturation (Mamessier *et al.*, 2011). Two immunotherapy strategies involving NK cells have been investigated: NSI with regards to endogenous NK cell activation and ACT with activated NK cells. Endogenous NK cell activation was executed using IL-2 and a marked increase in NK cells tumour infiltrates were noted, however, this increase was still not high enough to induce tumour regression (Albertsson *et al.*, 2003). The clinical trials for ACT of *in vitro* IL-2 activated NK cells into tumours produced disappointing results (Arai *et al.*, 2008; Iliopoulou *et al.*, 2010; Cho *et al.*, 2010; Geller *et al.*, 2011). A study by Re *et al.* (2006) used interleukin-2 (IL-2) and phytohaemagglutinin (PHA) to activate NK cells and increase NK cell induced apoptosis in immune-resistant tumours. This activation has been corroborated independently by Castriconi *et al.* (2004). The use of lymphodepletion and IL-2 therapy followed by NK ACT has proved ineffective so far in solid tumours clinically (Geller *et al.*, 2011).

It has been discovered that NK cells express ER α and ER β (Curran *et al.*, 2001) and while CD56^{dim} NK cells express PR-A and PR-B, CD56^{bright} NK cells lack PR expression (Arruvito *et al.*, 2008). This indicates that hormones as well as hormone-therapy drugs would affect NK

cell function. However, currently there has been no investigation into the effects of hormone-therapy on NK cell tumour populations or NK cell cytotoxicity in tumours.

The low numbers of NK cells in the TME indicates an immune evasion strategy (Georgiannos *et al.*, 2003; Macchetti *et al.*, 2006) that needs to be investigated as the presence of NK cells in tumours are associated with a better prognosis, which had been linked to NK cell activation (Albertsson *et al.*, 2003).

Rationale for the Study

Tumour cells have a reciprocal interaction with the tumour microenvironment. In hormone-dependent breast cancer this is particularly of importance with regards to hormone levels. Thus an *in vitro* investigation of the effects of the hormones 17β -oestradiol and progesterone as a simulation of an *in vivo* environment was deemed as necessary with regard to hormone-therapy strategies. Additionally, the presence of continuous treatment to mimic circulating hormones with regard to hormone-therapy is required. ER and PR expression is important in breast cancer prognosis and MUC1, although it is a relatively new biomarker in regards to breast cancer, is also implicated in prognostic significance. The quantification of the biomarkers expression is particularly useful when determining significant changes in expression levels and localisation.

Another critical component of the TME is immune cells and a great deal of research has been done with regards to tumour immunotherapy, particularly with regards to T-cells. However, although NK cells are rarely found in the TME, they are linked to a better prognosis. They are also hormone-responsive with ER and PR present. Therefore an investigation into the reciprocal interactions between hormone-therapy and NK cell immunotherapy in hormone-dependent breast tumour cells is required. Moreover, the effects of a hormone-simulated *in vivo* environment to determine if the presence of continuous endogenous hormones affects these combined therapeutics is necessary.

The hypothesis for this study was that hormone-therapy would increase the expression of all three biomarkers, and combined hormone-therapy and immunotherapy would reduce the expression of these biomarkers. It was further hypothesised that hormone-therapy and combined therapy would decrease MCF-7 cell proliferation. Combined therapy was also hypothesised to increase MCF-7 cell death, more so than hormone-therapy.

Aims and Objectives

This study thus sought to investigate the effects of hormone-therapy and combined hormone-therapy with immunotherapy on hormone-dependant breast cancer cells in a hormone simulated *in vitro* environment.

The objectives were as follows:

1. To investigate changes in the expression of the biomarkers ER α , PR and MUC1 in the MCF7 breast cancer cell line following hormone-therapy and combined hormone-therapy with immunotherapy.
2. To determine if hormone-therapy and combined hormone-therapy with immunotherapy has an effect on proliferation in the MCF7 breast cancer cell line.
3. To determine if hormone-therapy and combined hormone-therapy with immunotherapy induces apoptosis in the MCF7 breast cancer cell line.

MATERIALS AND METHODS

A stock of MCF-7 cells was first established for subsequent experimentation. Briefly, MCF-7 cells were plated, followed by the induction of cell cycle synchronisation. In order to simulate a hormonal environment, cells were incubated with appropriate concentrations of 17β -oestradiol and progesterone. Following this, cells were exposed to either hormone-therapy (Anastrozole or RU486) or combined hormone-therapy and hormones. They were then exposed to immunotherapy in the form of activated NK cells (Figure 2.1, Table 2.1). The MCF-7 cells were then subjected to the appropriate downstream applications: immunocytochemistry to examine the expression of PR, ER α , MUC1 and Ki67; or flow cytometry to assess the induction of apoptosis. For the immunotherapy, human blood was drawn to isolate the NK cells, therefore Human Ethics was obtained (M081036 (TN Augustine)) (Appendix A).

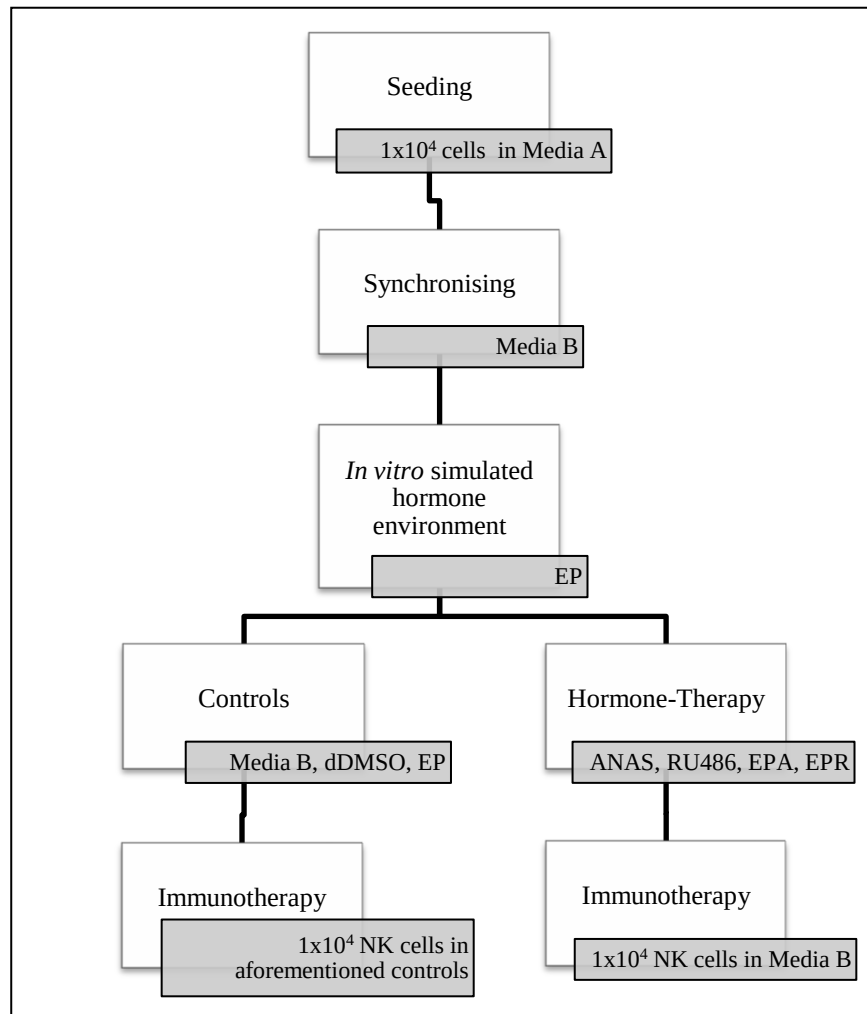


Figure 2.1. Flow diagram depicting a summary of the conditions the cells were exposed to for a hormone simulated environment and for the hormone-therapy and combined hormone-therapy with immunotherapy treatments.

Following adherence of cells in Media A, cells were synchronised in the cell cycle in Media B. A simulated *in vivo* microenvironment was created using the hormones 17 β -oestradiol and progesterone (EP). After the EP treatment, cells were either exposed to the hormone-therapy treatments: Anastrozole (ANAS) or RU486; or continue hormone stimulation with hormone-therapy: 17 β -oestradiol, progesterone and Anastrozole (EPA); and 17 β -oestradiol, progesterone and RU486 (EPR) (Table 2.1). For combined hormone-therapy and immunotherapy, hormone-therapy treated cells (with or without continued hormone stimulation) were incubated with NK cells. Appropriate controls for all treatments including Media B alone, dDMSO (diluent for all drugs and hormones) were conducted. Following treatment procedures cells were processed for immunocytochemistry or flow cytometry.

Cell Culture

MCF-7 cells were cultured in 25cm² Nunc culture flasks containing Media A (Dulbecco's modified Eagle's medium (DMEM) (Lonza, Bloemfontein, South Africa, BE12-604F) + 10% Foetal Calf Serum (FCS) (Biocom Biotech, Clubview, South Africa, A15101) + 0.1% Penicillin and Streptomycin (P/S) (Sigma-Aldrich, Aston Manor, South Africa, P3539) (Appendix B1) and incubated at 37°C and 5% CO₂. When subconfluent, these were subcultured using trypsin/ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, T4049) for detachment. Periodically the cells were stored in cryopreservation media DMEM + 60% FCS + 10% Dimethyl sulfoxide (DMSO) (Saarchem, Johannesburg, South Africa, 186500) (Appendix B2) at -80°C to provide a stock of MCF-7 cells for subsequent experimentation (Pawlak and Wiebe, 2007).

Cell Seeding

Each experiment was repeated three (n=3) times and cells were plated in duplicate for each assay. Glass coverslips (10mm round) were autoclaved and placed into labelled 24-well plates. Cryopreserved MCF-7 cells were thawed and washed twice in 1ml Phosphate Buffered Saline (PBS) (Appendix B3) followed by resuspension in 1ml Media A. To determine total cells, live cells and the percentage viability, the cell suspension was mixed 1:1 with Trypan Blue, then counted using a TC20 Automated Cell Counter (Biorad, Parkwood, South Africa). Each coverslip was plated with 1x10⁴ cells followed by 100µl Media A. Cells were incubated at 37°C and 5% CO₂ for 24 hours, for adherence to occur.

Thereafter, the cells were washed three times with PBS and incubated with 100µl of Media B (phenol red-free DMEM (Lonza, 12-917F) + 10% dextran coated charcoal stripped-FCS (DCC-FCS) + 0.1% P/S) (Appendix B4) at 37°C and 5% CO₂ for 48 hours to eliminate the effects of oestrogen and progesterone and like substances found in DMEM and FCS (Pawlak and Wiebe, 2007) and to synchronise the cells in the G1 phase of the cell cycle (Faivre *et al.*, 2005).

Hormone-Therapy

Anastrozole (Sigma-Aldrich, A2736) and RU486 (Sigma-Aldrich, M8046), as well as the hormones, progesterone (Sigma-Aldrich, P0130) and 17 β -oestradiol (Sigma-Aldrich, E2758), were used at a final concentration of 1 μ M. This concentration was previously shown to produce significant changes (Jeng *et al.* 1993; Pawlak and Wiebe, 2007). The drugs and the hormones were diluted in 0.01% DMSO and Media B (Appendix B7).

Following cell synchronisation, samples were washed three times with PBS and incubated with 100 μ l 1 μ M 17 β -oestradiol and 1 μ M progesterone (EP) at 37°C in 5% CO₂ for 24 hours to mimic the hormonal influences of the *in vivo* environment (Gil *et al.*, 2013). Cells were thereafter washed three times with PBS. Since preliminary research showed a significant change in ER α and MUC1 expression at 72 hours with Anastrozole or RU486 treatment (Gil *et al.*, 2013), cells were therefore incubated with 100 μ l 1 μ M of their respective treatments at 37°C and 5% CO₂ for 72 hours. The hormone-therapy treatments were as follows: Anastrozole (ANAS); RU486; 17 β -oestradiol, progesterone and Anastrozole (EPA); and 17 β -oestradiol, progesterone and RU486 (EPR) (Table 2.1).

Controls for the treatment groups were specifically selected to assess only the effects of the hormone-therapy drugs and immunotherapy. The control treatments were as follows: cell synchronisation medium (Media B); DMSO diluent (dDMSO); and 17 β -oestradiol and progesterone (EP) (Table 2.2). The Media B control was selected to provide a baseline without any treatment. The dDMSO was selected to assess the effect of the DMSO in which the hormones and drugs were dissolved. Lastly the EP control is a hormone control, to determine the effects of the hormones by themselves.

Table 2.1. Hormone-therapy treatments

MCF-7 cells were cultured in Media A for 24 hours followed by cell cycle synchronisation with Media B for 48 hours and the hormone pre-treatment (EP) for 24 hours. Afterwards the hormone-therapy treatments were performed for 72 hours. The treatments are Anastrozle (ANAS) and RU486, as well as these drugs with the addition of hormones (EPA and EPR).

Culture		Synchronisation		Pre-treatment		Treatment	
---	hrs	---	hrs	---	hrs	HT	hrs
Media A	24	Media B	48	EP	24	EPA	72
Media A	24	Media B	48	EP	24	EPR	72
Media A	24	Media B	48	EP	24	ANAS	72
Media A	24	Media B	48	EP	24	RU486	72

Table 2.2. The control treatments for hormone-therapy

MCF-7 cells were cultured in Media A for 24 hours followed by synchronisation with Media B for 48 hours and the hormone pre-treatment (EP) for 24 hours. Afterwards the control treatments were performed for 72 hours. The control treatments are: cell synchronisation control (Media B); DMSO diluent control (dDMSO); and 17 β -oestradiol and progesterone (EP) hormone control.

Culture		Synchronisation		Pre-treatment		Treatment	
---	hrs	---	hrs	---	hrs	Control	hrs
Media A	24	Media B	48	EP	24	dDMSO	72
Media A	24	Media B	48	EP	24	EP	72
Media A	24	Media B	48	EP	24	Media B	72

Immunotherapy

Magnetic Sorting of NK Cells

Blood (30ml) was drawn from seemingly healthy volunteers (n=6) by registered nurses into EDTA Vacutainers™ (BD Biosciences, Woodmead, South Africa, 367844). The blood was diluted with PBS (1:1), layered onto Ficoll-Paque Premium (GE Life Sciences, South Africa, 17-5442-03) at a ratio of 3:2 and then centrifuged for 30 min at 400xg. A white leukocyte layer was formed between the serum and Ficoll layers and this was transferred to a 10ml tube, followed by centrifuging at 400xg and washing with PBS. A sample of the cell suspension was diluted 1:1 with Trypan Blue and cell number and viability established using the TC20 Automated Cell Counter (average viability was 71%). The cell suspension was centrifuged again at 400xg for 5 min and the resulting cell pellet resuspended at 1×10^6 cells/ 80µl 10%FBS/PBS.

The cell suspension was thereafter incubated with 20µl APC-conjugated mouse anti-human NKp46 antibody (BD Biosciences, 558051), in the dark at room temperature for 15 min. Subsequently, cells were washed twice with Miltenyi Buffer (MB) (PBS + 0.5% BSA + 2mM EDTA) (Appendix B8) and resuspended at 1×10^7 cells/ 80µl MB. The cell suspension was thereafter incubated with 20µl anti-APC microbeads (Miltenyi Biotec, Cologne, Germany, 130-090-855) for 15 min in the dark at 4°C. The suspension was then washed twice with MB by centrifugation at 400xg for 5 min and resuspended at 1×10^7 cells/ 500µl MB for subsequent magnetic cell sorting.

For positive selection of NKp46⁺ cells, an MS column (Miltenyi Biotec, 130-042-201) was first placed in the magnetic field, primed with 3ml MB and then the cell suspension applied to the column followed by 3ml MB. This procedure was repeated three times. The column was then removed and 3ml MB added to the column reservoir. Positively selected NKp46⁺ NK cells were then flushed out into a collecting tube for subsequent viability assessment and activation.

The efficacy of this sorting technique for the NK cells was determined using flow cytometry by assessing the markers NKp46 and CD56 by TN Augustine in the same laboratory (Augustine *et al.*, 2015).

Activation of NK Cells

The magnetically sorted NK cell suspension was centrifuged at 400xg for 5 min. The cells were incubated 1:1 with Trypan Blue and viability and cell number assessed with the TC20 Automated Cell Counter (average viability was 71%). For activation, NK cells were incubated at 1×10^6 cells/100ul Activation Media A (Roswell Park Memorial Institute medium (RPMI) 1640 (Lonza, BE12-115F) + 0.1% P/S + 1 µg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich, L1668) + 0.8 ng/ml Interleukin-2 (IL-2) (Miltenyi Biotec, 130-093-901)) (Appendix B9) at 37°C and 5% CO₂ for 18 hours in a 96-well plate. Cell suspensions were then harvested, centrifuged at 400xg for 5 min and then incubated with Activation Media B (RPMI 1640 + 0.1% P/S + 0.8 ng/ml IL-2) (Appendix B10) for a further 30 hours (Domaica *et al.*, 2009). Subsequently NK cells were co-cultured with treated MCF-7 cells.

Co-culture of MCF-7 cells with NK cells

The activated NK cells were washed in PBS and resuspended in Media B. For determination of cell number and viability using the TC20 Automated Cell Counter, the NK cell suspension was mixed 1:1 with Trypan Blue (average viability was 61%). NK cells were added to the treated MCF-7 cells at a 1:10 ratio to simulate the low numbers of NK cells found in tumours. This co-culture was incubated at 37°C and 5% CO₂ for 4 hours in the present treatment media (Table 2.3). Two sets of controls were conducted: 1) hormone-therapy treatments alone (ANAS, RU486, EPA, EPR) and controls (Media B, EP, dDMSO), to determine the effect of the NK cells themselves; 2) controls as used for the hormone-therapy treatments with the addition of NK cells (Media B+NK, EP+NK and dDMSO+NK) (Table 2.4).

Table 2.3. Combined hormone-therapy and immunotherapy treatments

MCF-7 cells were cultured in Media A for 24 hours followed by synchronisation with Media B for 48 hours and the hormone pre-treatment (EP) for 24 hours. Afterwards the hormone-therapy treatments were performed for 72 hours followed by co-culture with activated NK cells (immunotherapy) for 4 hours.

Culture		Synchronisation		Pre-treatment		Treatment			
---	hrs	---	hrs	---	Hrs	Hormone-Therapy (HT)	hrs	Immunotherapy (IT)	hrs
Media A	24	Media B	48	EP	24	EPA	72	Activated NK cells	4
Media A	24	Media B	48	EP	24	EPR	72	Activated NK cells	4
Media A	24	Media B	48	EP	24	ANAS	72	Activated NK cells	4
Media A	24	Media B	48	EP	24	RU486	72	Activated NK cells	4

Table 2.4. The control treatments for combined hormone-therapy and immunotherapy

MCF-7 cells were cultured in Media A for 24 hours followed by synchronisation with Media B for 48 hours and the hormone pre-treatment (EP) for 24 hours. Afterwards the control treatments were performed. These included 1) hormone-therapy treatments alone and appropriate controls; 2) using the controls as determined for the hormone-therapy treatments with the addition of NK cells.

Culture		Synchronisation		Pre-treatment		Treatment			
---	hrs	---	hrs	---	Hrs	Hormone-Therapy (HT)	hrs	Immunotherapy (IT)	hrs
Media A	24	Media B	48	EP	24	EPA	72	---	4
Media A	24	Media B	48	EP	24	EPR	72	---	4
Media A	24	Media B	48	EP	24	ANAS	72	---	4
Media A	24	Media B	48	EP	24	RU486	72	---	4
Media A	24	Media B	48	EP	24	dDMSO	72	---	4
Media A	24	Media B	48	EP	24	EP	72	---	4
Media A	24	Media B	48	EP	24	Media B	72	---	4
Media A	24	Media B	48	EP	24	dDMSO	72	Activated NK cells	4
Media A	24	Media B	48	EP	24	EP	72	Activated NK cells	4
Media A	24	Media B	48	EP	24	Media B	72	Activated NK cells	4

Immunolocalisation of ER α , PR, MUC1 and Ki67

Following hormone-therapy and combined hormone therapy and immunotherapy treatments, immunolocalisation for the hormone receptors, ER α and PR; the glycoprotein MUC1 and the proliferation marker, Ki67, was conducted. Cells were washed thrice with PBS, fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, P6148) for 15 min and washed again. Cells were incubated in antigen retrieval buffer (ARB) (Appendix B11) at 95°C for 10 min, permeabilised in 0.25% TritonX-100 in PBS for 10 min at room temperature. Non-specific binding sites were blocked using 10% normal goat serum in 1% BSA/PBS for 30 min at room temperature. MCF-7 cells were double-labelled for ER α and MUC1 using 0.5 μ g/ml mouse anti-human MUC1 (Abcam, Pretoria, South Africa, ab28081) and 0.5 μ g/ml rabbit anti-human ER α (Abcam, ab31315). Cells were also double-labelled for PR and MUC1 using the aforementioned MUC1 antibody (0.5 μ g/ml) and 0.5 μ g/ml rabbit anti-human PR (Abcam, ab97801). Ki67 was localised alone with 1 μ g/ml rabbit anti-human Ki67 antibody (Abcam, ab15580). Cells were then incubated overnight at 4°C.

Thereafter, the cells were incubated at room temperature in the dark for 2 hours in 1 μ g/ml of each of the secondary antibodies: Alexa Fluor® 488 goat anti-rabbit (Life Technologies, Johannesburg, South Africa, A11008) and Alexa Fluor® 594 goat anti-mouse (Life Technologies, A11005) where applicable. The cells were incubated at room temperature in the dark for 5 min with 1:10000 DAPI (Sigma-Aldrich, 32670) and mounted using Fluoroshield (Sigma Aldrich, F6182). The slides were stored at 4°C until viewing.

The controls used were the following: a negative control (all antibodies omitted), primary antibody controls for MUC1, ER α , PR (the secondary antibody omitted), secondary antibody controls for Alexa Fluor® 488 and Alexa Fluor® 594 (the primary antibody omitted), and isotype controls for MUC1, ER α , PR and Ki67 (the primary antibody was incubated with either rabbit polyclonal IgG (Abcam, ab27478)(ER α , PR or Ki67) or mouse IgG3 (Abcam, ab18392)(MUC1) depending on the primary antibody, then added to the cells).

The Effect of Hormone-Therapy and Combined Therapy on Apoptosis

The flow cytometric Annexin V-FITC apoptosis detection Kit II (BD Biosciences, 556547) was used to determine the number of cells undergoing apoptosis. This is done using Annexin V-FITC to detect the phospholipid phosphatidylserine which translocates to the outer plasma membrane when a cell undergoes apoptosis. It also uses propidium iodide (PI) which enters the nucleus only when the plasma and nuclear membranes are breached.

The co-cultures of MCF-7 and NK cells were washed and the cells were detached using Accutase (Sigma Aldrich, A6964), a cell detachment solution of proteolytic and collagenolytic plant enzymes which ensures high cell viability and maintains cell surface marker expression for further analysis (Merten, 2010). The cell suspension was washed by centrifugation at 400xg for 5 min. The supernatant was then removed and the pellet resuspended in 1X binding buffer (0.01M Hepes (pH 7.4) + 0.14M NaCl + 2.5mM CaCl₂) at a concentration of 1×10^6 cells/ml. To the flow cytometric tubes, 100µl of the cell solution was added along with FITC Annexin-V and PI. The cell suspension was gently vortexed and incubated for 15 min in the dark at room temperature. The samples were analysed using the BD Biosciences LSRFortessa (Department of Surgery), and the data acquired using FACSDiva software. The data was subsequently analysed using FlowJo X 10.0.7r2.

The flow cytometer controls (for compensation and quadrant establishment) that were used were: MCF-7 cells incubated with 1) only FITC Annexin-V; 2) only PI and 3) with no antibodies (See Appendix C). The apoptosis assay controls that were used were: 1) a negative control (Media B); 2) an apoptosis positive control (camptothecin (CPT)); and 3) a DMSO diluent control (dDMSO control as previously discussed). The CPT control was used as it stimulates apoptosis via the mitochondrial pathway, it was used at a concentration of 5µM (Hingorani *et al.*, 2011).

Acquisition and Analysis of Images

During the stages of culture and treatment, phase contrast images were taken using an Olympus IX51 Inverted Microscope, an Olympus XC10 camera and CellSens software.

Cells were viewed under an Olympus IX51 Inverted Fluorescent Microscope with U-MWIB2 (green Alexa Fluor 488), U-MIY2 (red Alexa Fluor 594) and U-MNU2 (blue DAPI) filters. Images acquired were designated DAPI, MUC1, PR, ER or Ki67. The MUC1, ER and PR images were obtained at 20x or 40x magnification, and the Ki67 images were obtained at 10x magnification. Fluorescence was captured individually as grayscale images and colour representation generated as per fluorescence channels. Images showing Alexa Fluor 488 (green) or Alexa Fluor 594 (red) were overlaid with DAPI (blue) to illustrate nuclear localisation of these markers. Furthermore, where MCF-7 cells were double-labelled i.e. MUC1 and ER; MUC1 and PR, the colour images were overlaid to identify colocalisation of the markers.

To obtain quantitative data, these images were analysed using CellProfiler (Carpenter *et al.*, 2006). Two pipelines were created in CellProfiler for the analysis of the different types of markers. Specifically, Pipeline A was used to analyse the expression of MUC1, ER α and PR (Figures 2.2, 2.3), and Pipeline B was used to analyse the expression of Ki67 (Figures 2.4, 2.5). Pipeline A thus calculated mean intensity values for ER α , PR and MUC1 expression in the nucleus and the cytoplasm for comparison purposes. Pipeline B was designed to calculate the percentage of the nuclei that were Ki67 positive (Ki67⁺).

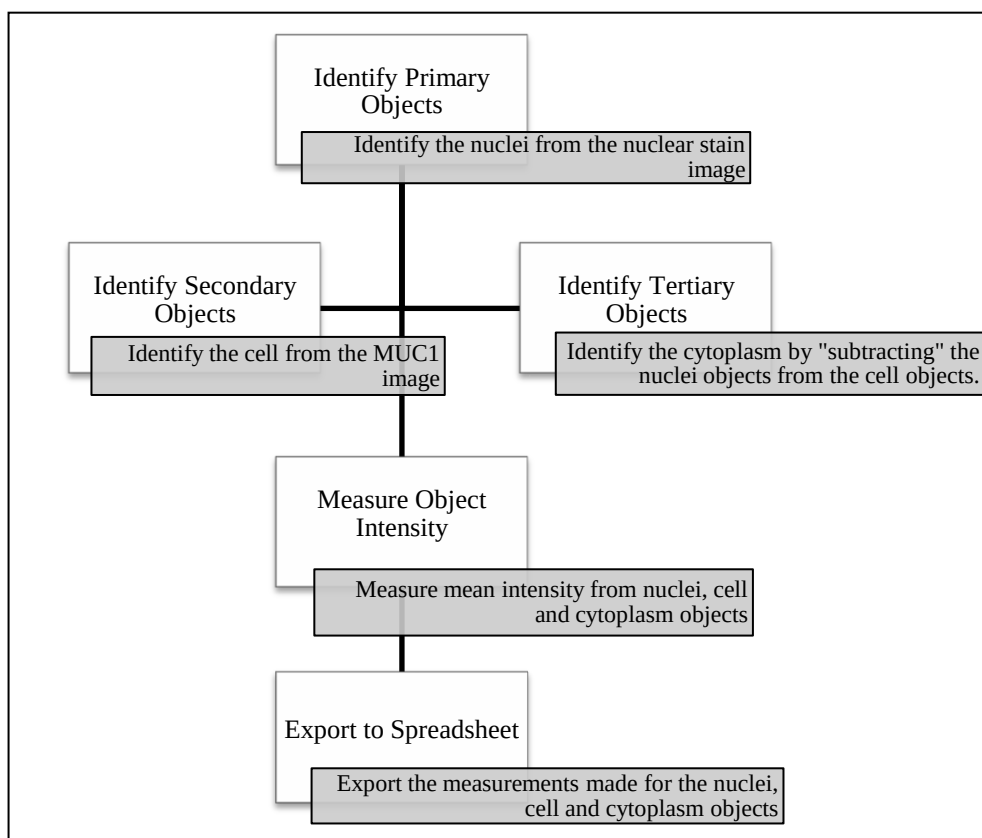


Figure 2.2. Pipeline A for biomarker analysis using CellProlifer

The pipeline used to analyse both the MUC1+ER α images as well as the MUC1+PR images producing the values mean intensity (the average pixel intensity within the object) for each of the cell, nuclei and cytoplasm objects.

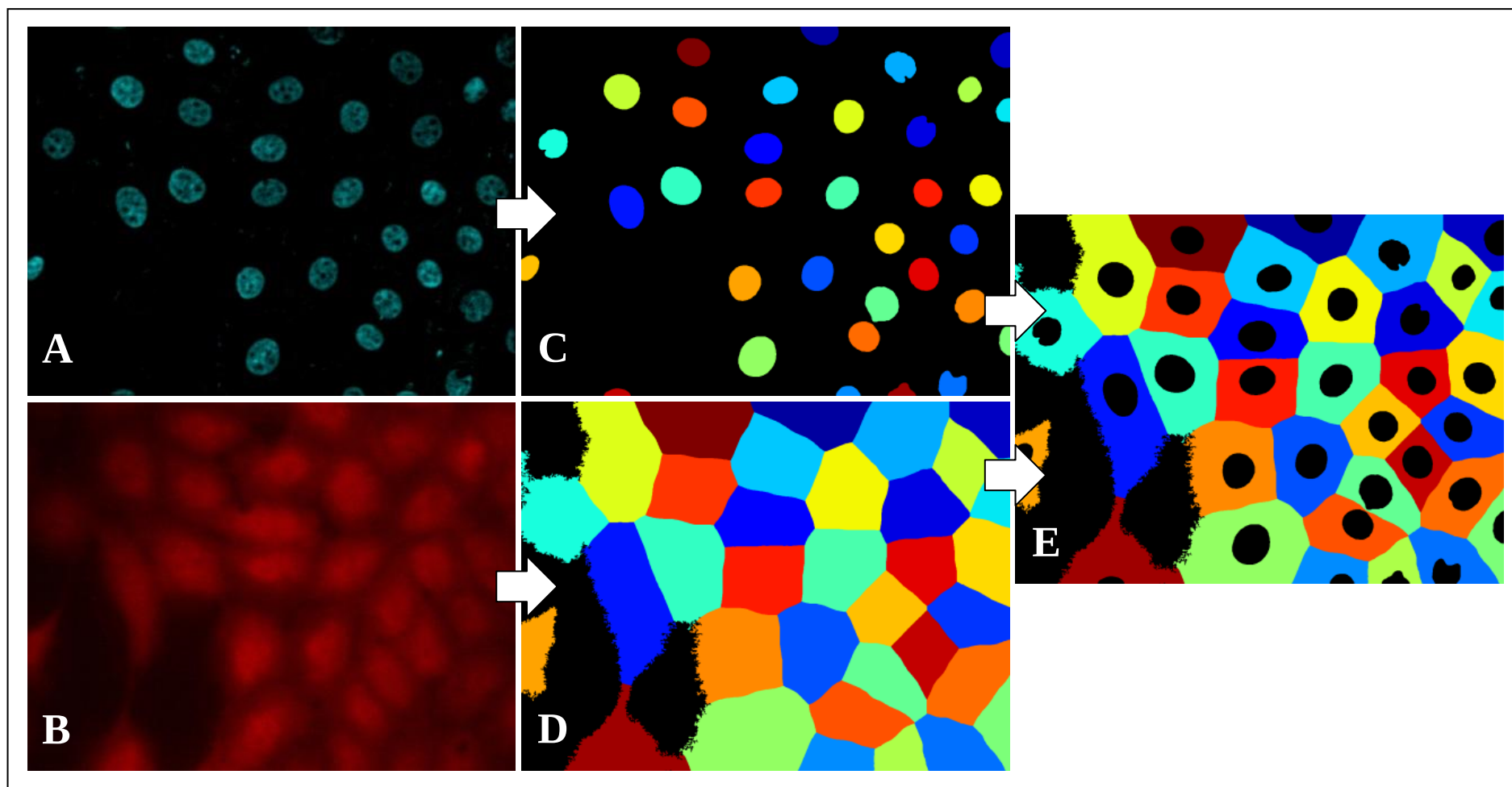


Figure 2.3. An illustrative overview of the CellProfiler pipeline created to determine the three objects measured (nucleus, cell and cytoplasm)

A: Nuclei stained with DAPI (blue). **B:** Immunolocalisation of MUC1 with Alexa Fluor 594 (red cytoplasmic and nuclear staining). **C:** Area occupied by individual nuclei as determined by CellProfiler. **D:** Area occupied by the entire cell as determined by CellProfiler. **E:** Area occupied by the cytoplasm of the cell only (C is subtracted from D)

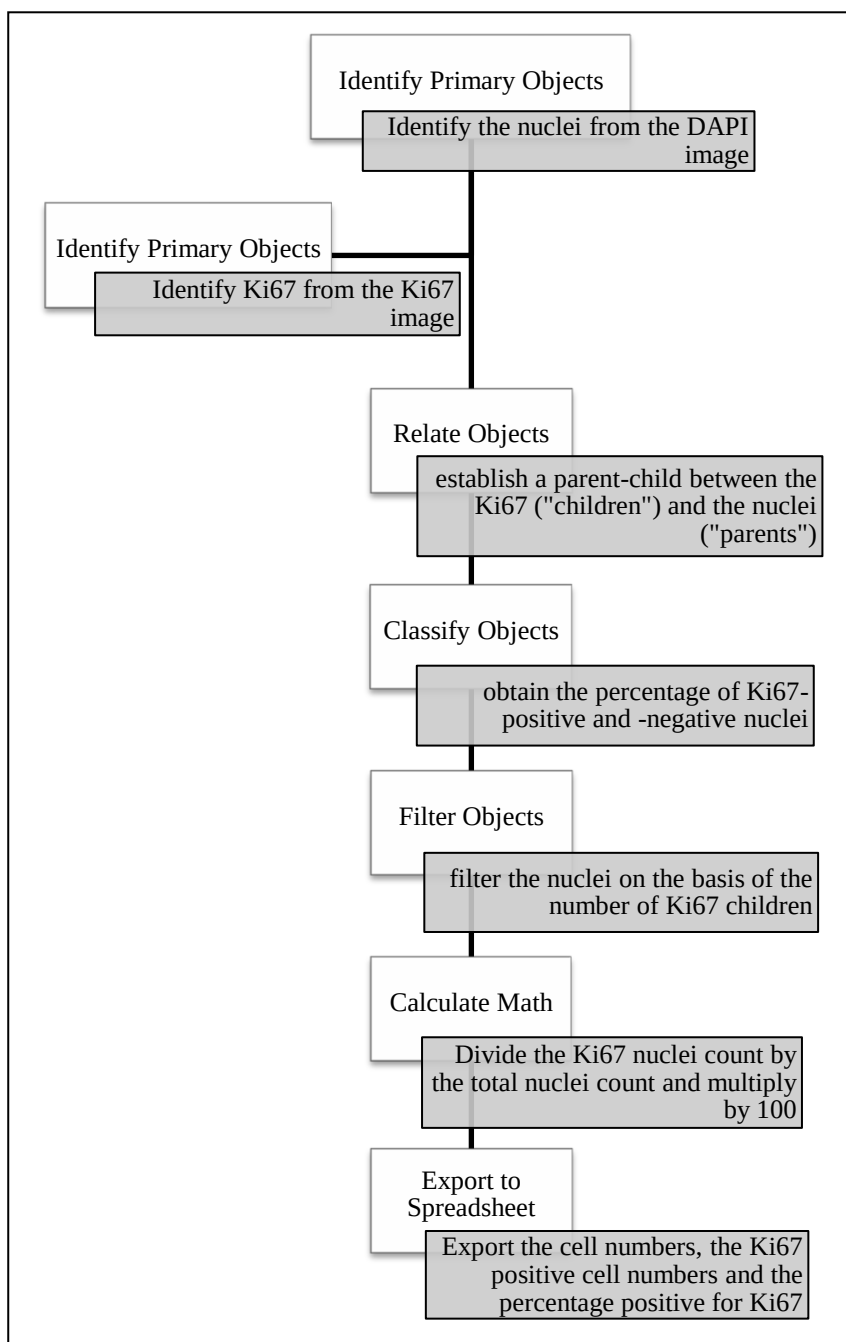


Figure 2.4. Pipeline B for Ki67 analysis using CellProfiler

A CellProfiler pipeline used to analyse the Ki67 slides and produce the percentage of nuclei that are Ki67 positive.

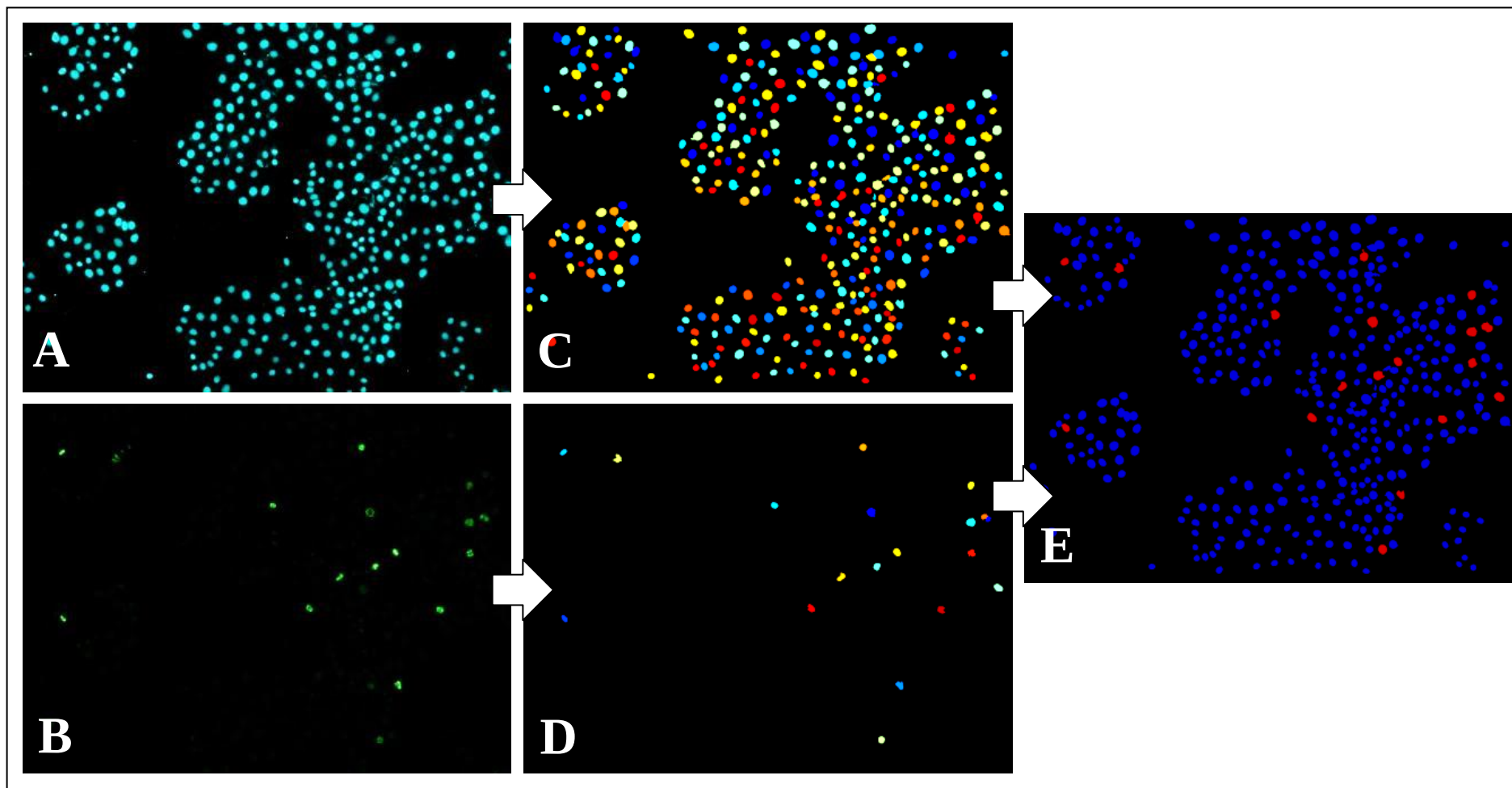


Figure 2.5. An illustrative overview of the CellProfiler pipeline created to determine the percentage of nuclei positive for Ki67

A: Nuclei stained with DAPI (blue). **B:** Immunolocalisation of Ki67 with Alexa Fluor 488 (green). **C:** Area occupied by individual nuclei as determined by CellProfiler. **D:** Area occupied by Ki67 as determined by CellProfiler. **E:** Composite image showing Ki67⁺ nuclei (red areas, Ki67, associated with blue, nuclei) produced by CellProfiler.

Statistical Analysis

Statistical analysis was conducted using the statistics and analytics software package PAST (Paleontological Statistics), a free data analysis package which is used for common statistical plotting and testing. Normality tests (Shapiro-Wilk, Jarque-Bera, χ^2 and Anderson-Darling) were conducted to determine normality of the data with significance set at $p=0.05$. Where PAST was unable to perform a normality test on a data set, due to a floating point error, the software package Statistical Analysis System (SAS) was used.

The biomarker data was found to be not normally distributed ($p<0.05$) (Appendix D, Tables D2, D8 and D14) and thus the following tests were employed: non-parametric Kruskal-Wallis test and Mann-Whitney test. Significance was set at $p=0.001$ and for more accurate assessment, Bonferroni corrected p -values were used. Spearman's correlation was also used to determine correlation values for the biomarkers, MUC1 and ER α , and MUC1 and PR.

The Ki67 data was found to be normally distributed ($p>0.05$) (Appendix D, Table D21) and thus the parametric one-way analysis of variance (ANOVA) test was used and pairwise comparisons were performed using Tukey's (default of PAST). Significance was set at $p=0.05$.

The flow cytometric data used to analyse the expression of phosphatidylserine and propidium iodide staining, was not normally distributed ($p<0.05$) (Appendix D, Table D24) and thus the non-parametric Kruskal-Wallis test was employed. Significance was set at $p=0.05$.

The software package Statistica 12.5 was used to produce the bar graphs and correlation graphs.

RESULTS

General Morphology of MCF-7 Cells

The MCF-7 cell line is derived from a pleural effusion of a metastatic mammary carcinoma; it is a hormone-dependent cancer (ER and PR positive) and is also positive for MUC1 (Zaretsky *et al.*, 2006). These cells, seeded as a single cell suspension, form plaques as they attach to the bottom of the culture flask and become a monolayer. The monolayer retains some of the characteristics of its epithelial origin. Cells predominantly display a polygonal shape with a centrally placed nucleus containing multiple nucleoli (Figure 3.1A). Some cells exhibit a mesenchymal morphology (Dhasarathy *et al.*, 2007). These cells are smaller and more elongated, with a smaller nucleus and attenuated cytoplasm. The distinction between the two cell types is easily seen (Figure 3.1B), with the typical mesenchymal-like morphology commonly indicating a less differentiated phenotype. As the monolayer becomes more confluent some of the cells form rosettes (Figure 3.1B). Rosettes are typically associated with duct formation (Soule *et al.*, 1973), however no lumen formation was noted (Figure 3.1B).

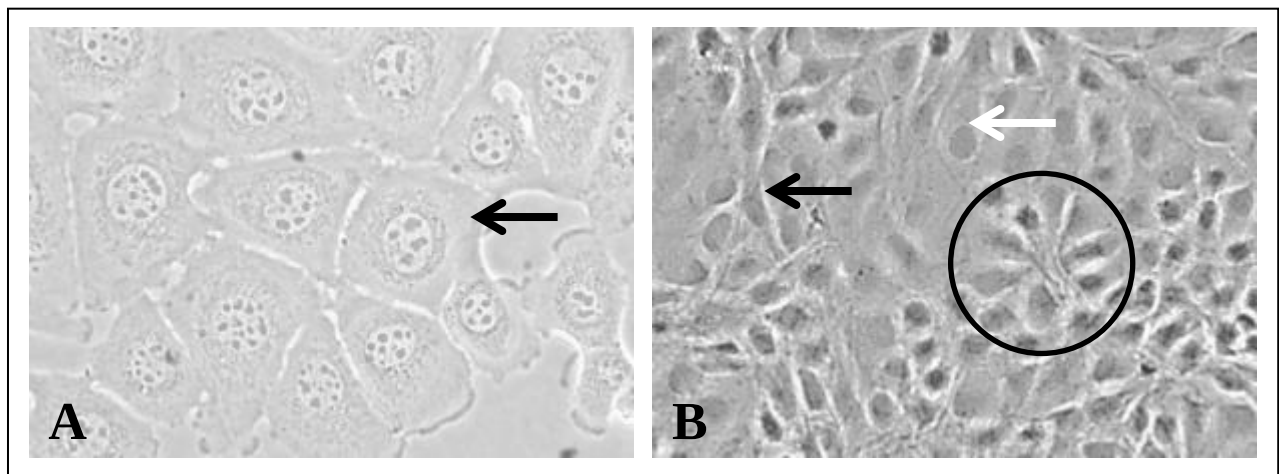


Figure 3.1. Representative photomicrographs showing the morphology of MCF-7 cells under normal culture conditions

A: MCF-7 cells forming plaques illustrating the large polygonal shaped cells with a centrally placed nucleus containing multiple nucleoli (black arrow). Phase contrast microscopy, 40x magnification. **B:** Subconfluent MCF-

The Effect of Hormone-Therapy and Combined Therapy on MCF-7 Cell Morphology

The general morphology of cells in the control treatment groups (Media B, dDMSO and EP) did not differ drastically from each other showing large polygonal cells, small elongated cells and rosettes (Figure 3.2). The cells in the Media B control treatment group appeared to be more densely packed and there also appeared to be more rosettes (Figure 3.2A). However, EP treatment appeared to induce an increase in the number of large polygonal cells (Figure 3.2B).

Hormone-therapy (HT) (Figure 3.3) or combined hormone-therapy and immunotherapy treatments (HT+IT) (Figure 3.4) resulted in the majority of cells displaying a large polygonal shape, indicative of a more differentiated phenotype, and the presence of small elongated cells, indicative of a more mesenchymal-like phenotype. Specifically under NK cell mediation, RU486 induced prominent plaque formation and fewer rosettes as compared to the other treatments in both the HT (Figure 3.3) and HT+IT groups (Figure 3.4).

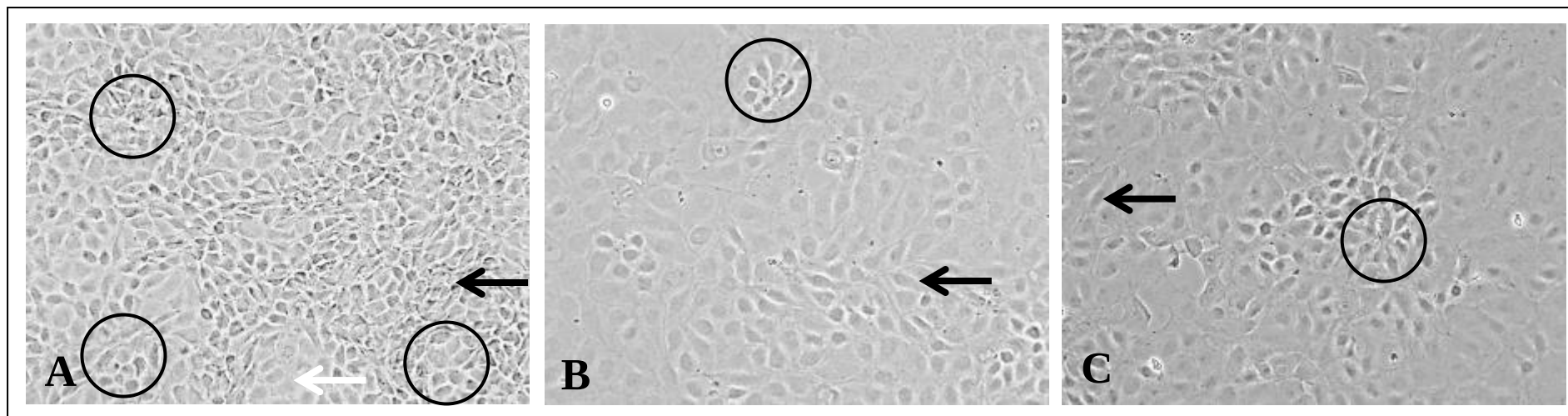


Figure 3.2. Representative photomicrographs showing the effects of control treatments on MCF-7 cell morphology

Control treatment groups: **A:** Media B. **B:** EP. **C:** dDMSO. Phase contrast microscopy, 20x magnification.

Small, elongated cells with a smaller nucleus and less cytoplasm (black arrow) and larger, polygonal cells (white arrow) are noted. Some cells show rosette formation occurring (encircled). The EP treatment appears to have induced more large polygonal cells and the media B treatment appears to have induced more rosette formation.

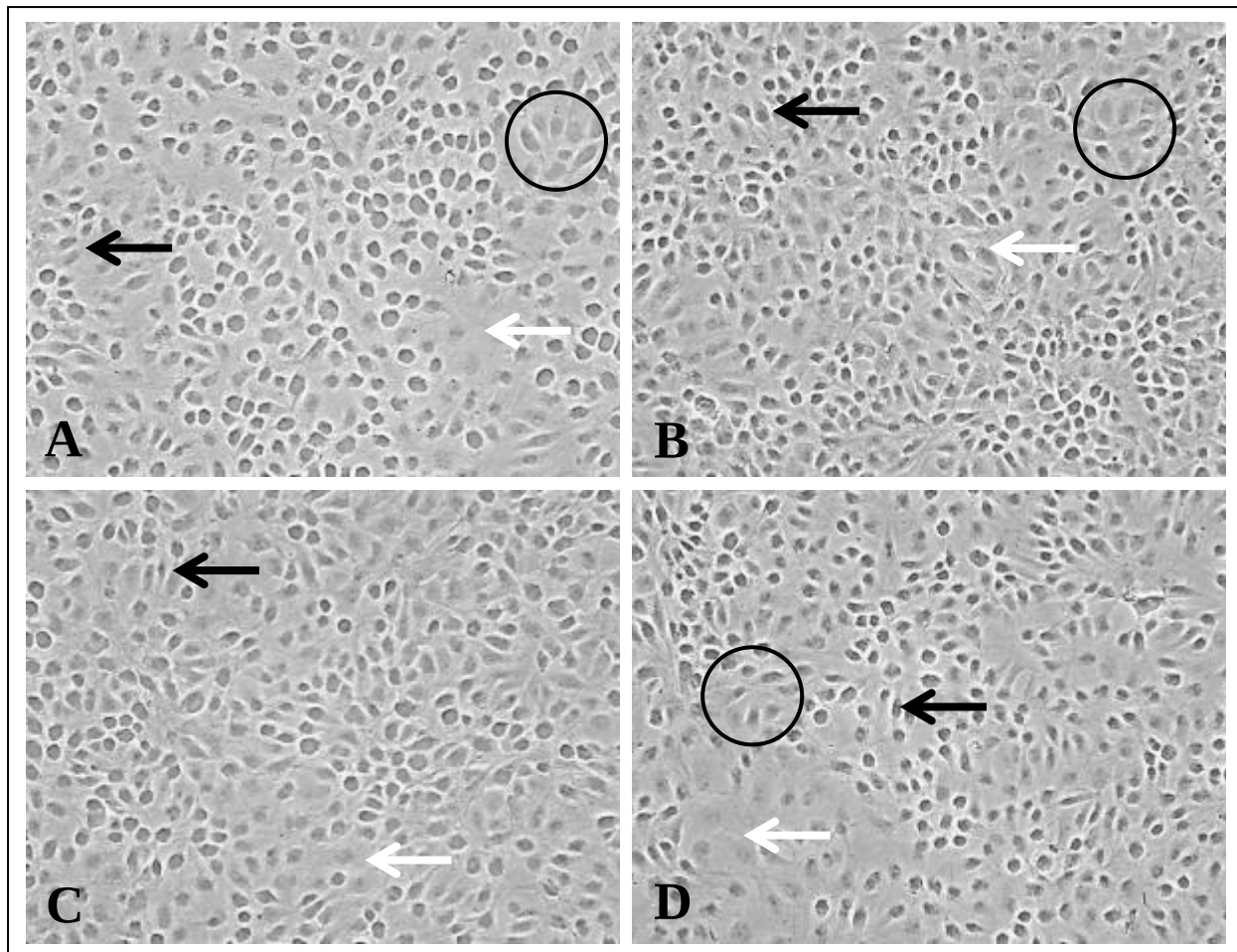


Figure 3.3. Representative photomicrographs showing the effects of hormone-therapy on MCF-7 cell morphology

Treatment groups: A: ANAS. B: RU486. C: EPA. D: EPR. Phase contrast microscopy, 20x magnification.

A heterogeneous population of MCF-7 cells are noted, with some cells displaying a smaller, elongated morphology (black arrow) and other cells showing a larger, more polygonal morphology with indistinct cell boundaries (white arrow). Note rosette formation (encircled).

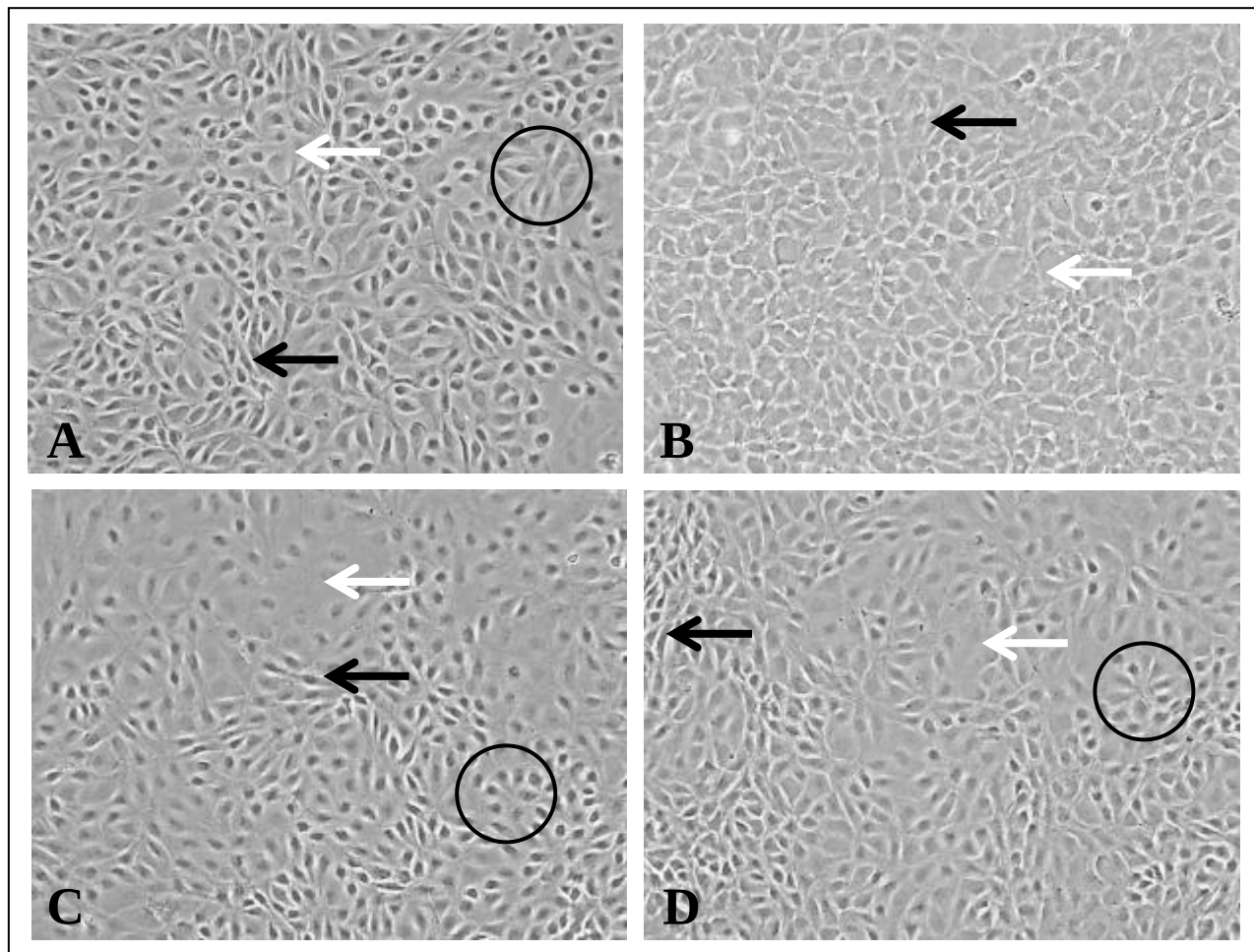


Figure 3.4. Representative photomicrographs showing the effects of combined hormone-therapy and immunotherapy on MCF-7 morphology

Treatment groups: **A:** ANAS+NK. **B:** RU486+NK. **C:** EPA+NK. **D:** EPR+NK. Phase contrast microscopy, 20x magnification. In most treatment groups, a mixed morphology is noted, with small elongated cells (black arrow), and large polygonal cells (white arrow). Rosette formation was also noted (encircled). Under NK cell mediation, Anastrozole appeared to induce more small cells, with a mesenchymal-like morphology (A). The addition of hormones resulted in an increase in the number of polygonal cells (C). The RU486+NK treatment group appears to have fewer mesenchymal-like cells (B), with cells having formed a plaque with distinct cell boundaries and no discernible rosettes. The addition of hormones, appears to have resulted in more cells displaying a mesenchymal-like morphology.

Immunolocalisation of MUC1, ER α and PR

The ICC technical controls were the following: negative control, primary antibody controls for MUC1, ER α and PR, secondary antibody controls for Alexa Fluor® 488 and Alexa Fluor® 594, and isotype controls for MUC1, ER α and PR. These ICC controls showed no fluorescence (see Appendix C, Figure C1) indicating that there was no background fluorescence and no autofluorescence. This indicates the specificity of the antibodies and the effectiveness of the ICC procedure.

Following hormone-therapy (HT) and combined hormone-therapy and immunotherapy (HT+IT) of the MCF-7 cells, double-labelling with MUC1 and ER α , or MUC1 and PR was conducted. The ER α and PR proteins were visualised with the Alexa Fluor 488 antibody producing a green fluorescence. The MUC1 protein was visualised using the Alexa Fluor 594 antibody, producing a red fluorescence. The nuclei were visualised using DAPI, producing a blue fluorescence. Fluorescence was captured individually as grayscale images and colour images generated thereafter. For qualitative analysis colour images were overlaid to show fluorescence in both the nuclear and cytoplasmic compartments. Where either PR or ER α localisation (green) overlapped the DAPI-stained nucleus (blue), a lighter blue-green nucleus was noted. For quantitative analysis, images were analysed with CellProfiler using a pipeline that determined the mean fluorescence intensity (MFI) of each immunolocalised protein for the nucleus and the cytoplasm separately.

The Effects of Hormone-Therapy and Combined Therapy on PR Immunolocalisation

For all controls and treatments performed on the MCF-7 cells, it was observed that there were more cells exhibiting a polygonal morphology as opposed to mesenchymal morphology. Cells exhibiting the mesenchymal morphology presented a high nuclear and cytoplasmic fluorescent intensity (Figure 3.5) while the polygonal-shaped cells vary in PR intensity between the nucleus and cytoplasm, depending on control and treatment groups.

PR is visible in both the cytoplasm and the nucleus of the cells in all control groups and treatment groups. The Media B control (cell cycle synchronisation media) induced the highest cytoplasmic PR fluorescent intensity (Figure 3.5A) compared to cells in the dDMSO control group and EP control group. The latter control groups demonstrated an increase in PR fluorescent intensity in the majority of cell nuclei (Figure 3.5B, C). Increased PR nuclear expression is noted where overlap between the DAPI (blue fluorescence) and Alexa Fluor 488 (green fluorescence) results in a lighter blue region (Figure 3.5B). Within these control groups, cells treated with Media B exhibited the highest cytoplasmic fluorescent intensity and the cells treated with hormones (EP) exhibited notable reduction in cytoplasmic PR expression. In the majority of the cells analysed for all the treatments, high nuclear expression was observed in the more mesenchymal-like cells and high perinuclear expression in the more polygonal cells; however, this may be a function of cell shape and size.

Cells in the hormone-therapy (HT) groups (ANAS, RU486, EPA and EPR) all exhibited differing levels of cellular PR localisation between treatment groups (Figure 3.6). All HT simulations induced a high fluorescent intensity in the nucleus as indicated by the light blue-green colour (Figure 3.5) due to obscuring of the DAPI stain. Cells treated with Anastrozole (ANAS), exhibited low PR cytoplasmic intensity with a higher nuclear presence (Figure 3.6, A) compared to the diluent control (Figure 3.5C). With the addition of hormones, no variation in cytoplasmic intensity was discernible in EPA treated cells as compared to those treated with Anastrozole alone (Figures 3.6A, C). Cells treated with RU486 showed a high fluorescent intensity in the perinuclear area as well as the nucleus (Figures 3.6B). However, EPR treatment induced a reduction in cytoplasmic fluorescent intensity compared to treatment with RU486 alone (Figures 3.6D). Notably, in all treatment groups with the exception of RU486, cytoplasmic PR staining appeared reduced compared to the Media B and dDMSO controls (Figure 3.5).

Cells in the hormone-therapy and immunotherapy (HT+IT) groups (ANAS+NK, RU486+NK, EPA+NK and EPR+NK) also exhibited variations in PR fluorescent intensity (Figure 3.7), with notable nuclear PR fluorescence. The ANAS+NK treated cells exhibited low cytoplasmic fluorescent intensity with variable fluorescent intensity of the nucleus (Figure 3.7A). The RU486+NK treatment induced a slight increase in nuclear fluorescent intensity as compared to the cytoplasm (Figure 3.7B); however, overall PR fluorescent intensity was remarkably low compared to all HT+IT groups. The EPA+NK and EPR+NK treatments induced an intermediate to high cytoplasmic PR fluorescent intensity with a high intensity in the nucleus (Figure 3.7C, D) compared to both hormone-therapy drugs alone (ANAS+NK; RU486+NK).

When comparing the HT to the HT+IT treatment groups, Anastrozole alone (ANAS) and with hormones (EPA) induced a higher fluorescent intensity in the perinuclear region as opposed to the nucleus in cells as in the respective immunotherapy groups (Figures 3.6A, C; Figures 3.7A, C). Cells in the EPR and EPR+NK groups responded similarly, with a low cytoplasmic PR fluorescent intensity and higher nuclear fluorescent intensity (Figure 3.6D; Figure 3.7D). Conversely, there was a higher fluorescent intensity in cells treated with RU486 alone compared to RU486-treated cells cultured with NK cells (Figure 3.6B, Figure 3.7B). Qualitative assessment thus indicates that PR fluorescent intensity and localisation varies with treatment, notably being under considerable influence from RU486. Moreover the definitive reduction in cytoplasmic PR fluorescence with the addition of NK cells to RU486-treated cells indicates that cells may modulate their PR expression not only to treatment but to immune influence as well.

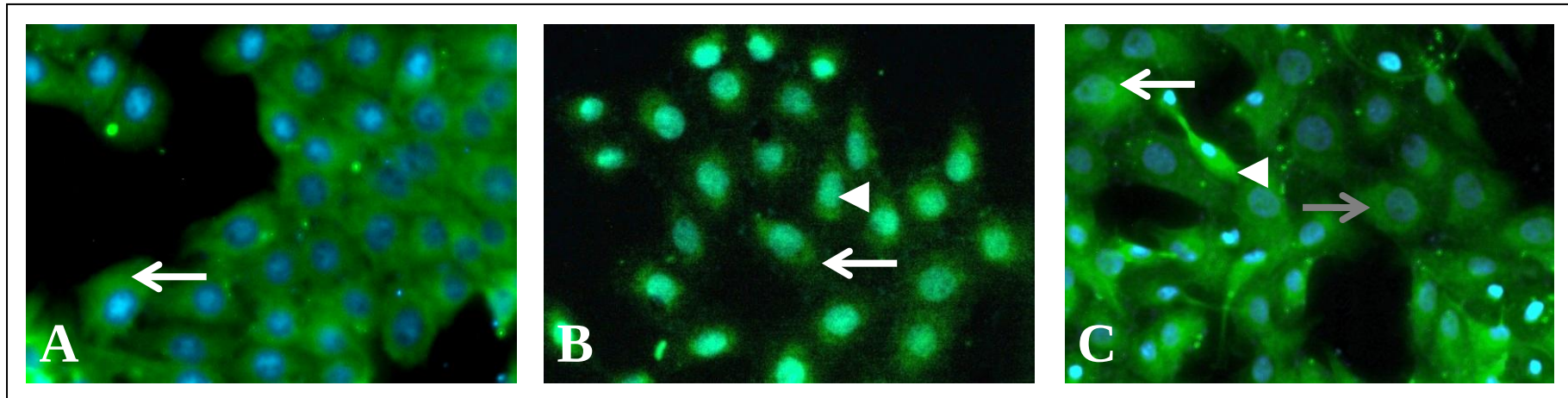


Figure 3.5. Representative photomicrographs showing the effects of control treatments on PR expression in MCF-7 cells

Control treatment groups: **A:** Media B. **B:** EP. **C:** dDMSO. Fluorescent microscopy, 40x magnification. **A:** Media B treatment results in high cytoplasmic PR intensity with a comparative increase in intensity in the perinuclear region (arrow). **B:** EP treated cells exhibit overall low PR intensity expression with an increased intensity (compared to the cytoplasm) in the nucleus (arrowhead) and additionally increased intensity in the perinuclear area in a few cells (arrow). **C:** The DMSO diluent control induced a high nuclear fluorescent intensity with an intermediate cytoplasmic intensity in the more polygonal cells (white arrow) and a high cytoplasmic intensity in the more mesenchymal-like cells (arrowhead), and a few cells demonstrate a higher intensity in the perinuclear area (grey arrow).

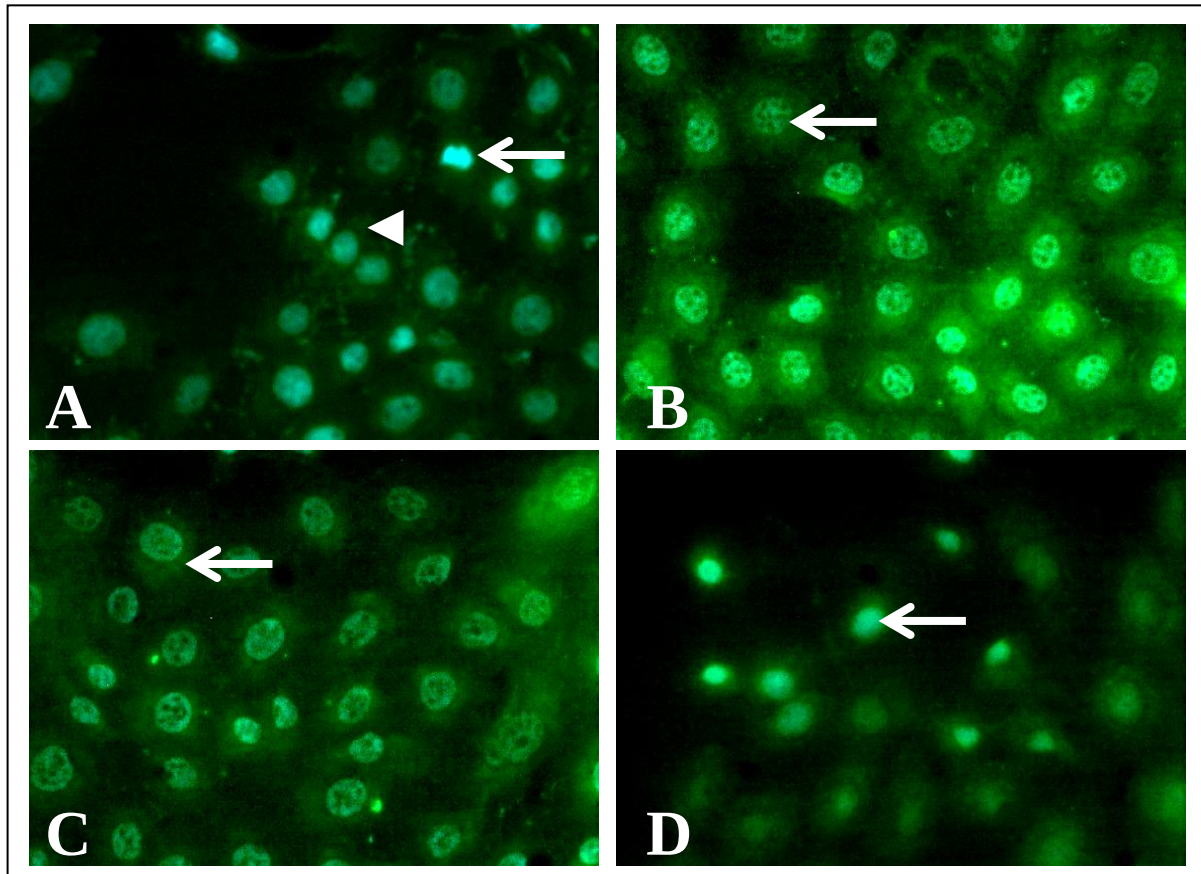


Figure 3.6. Representative photomicrographs showing the effects of hormone-therapy on PR expression in MCF-7 cells

Treatment groups: **A:** ANAS. **B:** RU486. **C:** EPA. **D:** EPR. Fluorescent microscopy, 40x magnification. **A:** The cells treated with ANAS exhibited a low PR cytoplasmic intensity (arrowhead) and higher nuclear intensity (arrow). **B:** RU486 induced a high nuclear PR fluorescent intensity in most of the cells, with a lower intensity in a few cells (arrow) as well as an increased PR concentration in the perinuclear region in some cells (arrowhead). **C:** EPA treated cells show a low cytoplasmic PR fluorescent intensity, with a higher nuclear and perinuclear fluorescent intensity (arrow). **D:** The EPR treatment induced a low cytoplasmic fluorescent intensity and a comparatively higher nuclear fluorescent intensity (arrow).

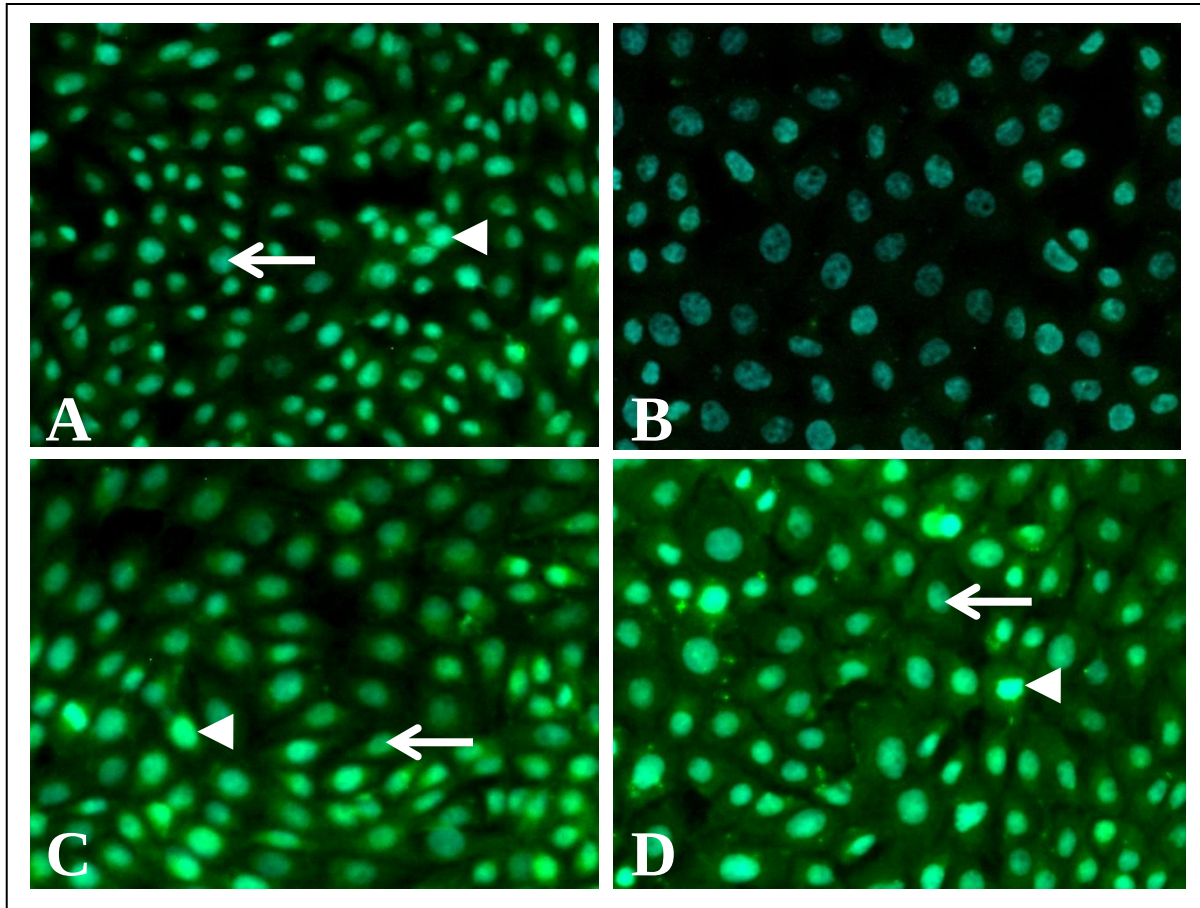


Figure 3.7. Representative photomicrographs showing the effects of combined hormone-therapy and immunotherapy on PR expression in MCF-7 cells

Treatment groups: **A:** ANAS+NK. **B:** RU486+NK. **C:** EPA+NK. **D:** EPR+NK. Fluorescent microscopy, 20x magnification (digitally magnified a quarter of the image). **A:** The majority of cells in the ANAS+NK treatment group show a low to intermediate nuclear fluorescent intensity (arrow), with a few cells displaying high nuclear fluorescent intensity (arrowhead). There is also a low cytoplasmic PR fluorescent intensity in the cells. **B:** RU486+NK treatment induced a very low fluorescent intensity of PR in both the nucleus and the cytoplasm. **C:** The EPA+NK treatment induced a high PR intensity in the nucleus of some cells (arrowhead), and a low to intermediate intensity in the majority of the cells (arrow). There is also a low to intermediate PR fluorescent intensity in the cytoplasm of the cells. **D:** Cells treated with EPR+NK exhibit a low to intermediate nuclear fluorescent intensity in the majority of the cells (arrow), and a few cells with a high nuclear fluorescent intensity (arrowhead). An intermediate cytoplasmic intensity is also seen with this treatment.

Images were then analysed further using CellProfiler. For quantitative analysis of PR expression, the normality tests Shapiro-Wilk, Jarque-Bera and χ^2 were first performed on the data produced by CellProfiler, with significance set at $p=0.05$. These include total cellular expression, nuclear expression and cytoplasmic expression. Most of the data sets were not normally distributed ($p<0.05$) (Appendix D, Table D2) thus the non-parametric Kruskal-Wallis one-way analysis of variance was used to compare each data set and pairwise comparisons were performed between and within the data sets with the Mann-Whitney test, with significance for both set at $p<0.001$ (Appendix D, Tables D3-D6).

When assessing PR expression in the control treatment groups, considerable variation was noted. The DMSO diluent control (dDMSO) induced a significant increase ($p<0.001$) in PR mean fluorescent intensity (MFI) as compared to the Media B control in both the nucleus and cytoplasm (Figure 3.8). This indicates that DMSO, even as a diluent, has an effect on PR expression. The EP hormone control induced a nuclear and cytoplasmic PR MFI that was significantly decreased ($p<0.001$) to that of both other controls. Across all the control groups (Media B, dDMSO and EP), there was a significant increase ($p<0.001$) in PR MFI in the nucleus compared to the cytoplasm. These results bear out qualitative observations; however, quantitative analysis indicates that nuclear expression in the EP group is significantly reduced compared to the Media B control group which contradicts qualitative observations (Figure 3.5A, B).

Importantly, since hormones and drugs were all dissolved in DMSO, to isolate their effects from that of the DMSO diluent, all subsequent data analysis of the effects of hormone-therapy, and combined hormone-therapy and immunotherapy on biomarker expression used the dDMSO control instead of the Media B control as the comparator group. The EP control group remained as an additional hormone control group for combined hormone and drug treatments (EPA and EPR). Furthermore, in addition to these controls when comparing the effects of hormone therapy alone against hormone-therapy and immunotherapy, the additional control comparator groups, dDMSO+NK and EP+NK, were also employed.

The Effects of Anastrozole on PR MFI

Firstly, differences between nuclear and cytoplasmic PR expression was assessed within each treatment group. Treatment with Anastrozole (ANAS) showed no significant difference in PR MFI between the nuclear and cytoplasmic compartments (Appendix D, Table D3). However, with the addition of hormones (EPA) or NK cells with and without hormones (ANAS+NK, EPA+NK) there was a distinct reduction in PR cytoplasmic localisation with a corresponding increase in nuclear localisation ($p < 0.001$) (Appendix D, Tables D1, D3 and D4). These results are borne out by the qualitative observations (Figure 3.5 - Figure 3.7); however, the quantitative results highlight shifts in expression more accurately between nuclear and cytoplasmic compartments within treatment groups.

When comparing Anastrozole-induced changes in overall MFI to the dDMSO control, Anastrozole alone (ANAS) caused a significant decrease in both nuclear and cytoplasmic PR MFI (Figure 3.9). The reduction in cytoplasmic PR staining was easier to visualise compared to nuclear alterations (Figure 3.5C, Figure 3.6A). The quantitative reduction in PR cytoplasmic MFI was mimicked with the addition of hormones (EPA) (Figure 3.9) with respect to the diluent control (dDMSO), the hormone control (EP) and Anastrozole treatment itself (ANAS). However, visually this was difficult to interpret with fluorescent images alone (Figure 3.5, Figure 3.6). The quantitative results clearly portray that within a hormonal environment Anastrozole reduces PR expression, more so than Anastrozole treatment alone.

With the addition of immunotherapy (combined hormone-therapy and immunotherapy) (Figure 3.10), Anastrozole (ANAS+NK) remained significantly reduced ($p < 0.001$) compared to the diluent control (dDMSO) in both nuclear and cytoplasmic PR expression. These results confirm qualitative observations (Figure 3.5C, Figure 3.7A). The addition of hormones (EPA+NK) reflected the same trend both quantitatively (Figure 3.10) and qualitatively (Figure 3.5C, Figure 3.7C). This PR MFI is further significantly reduced compared with the hormone control (EP). Compared to Anastrozole itself (ANAS+NK), the PR MFI was significantly increased in the nuclear compartment, and decreased in the cytoplasmic compartment in the EPA+NK group (Figure 3.10). This suggests that NK mediation of hormone-therapy differentially affects cellular compartments. It is only with comparison of hormone-therapy alone and combined hormone-therapy and immunotherapy that the full picture emerges.

Thus the PR MFI between hormone-therapy and combined hormone-therapy and immunotherapy was compared. It was noted that NK cell mediation significantly reduced PR expression in both the DMSO (dDMSO vs dDMSO+NK) and EP controls (EP vs EP+NK) (Figure 3.11). When Anastrozole, either with hormones (EPA) or without (ANAS), was compared to its counterpart with NK cells (EPA+NK, ANAS+NK), it was found that NK cell interaction caused a significant decrease ($p < 0.001$) in PR MFI in both the nucleus and the cytoplasm (Figure 3.11). This was also noted qualitatively (Figure 3.7). These results highlight that combined Anastrozole hormone-therapy with immunotherapy induces decreased PR cellular expression, as compared with hormone-therapy alone.

The Effects of RU486 on PR MFI

We first assessed nuclear and cytoplasmic PR alterations within each treatment group. RU486 alone showed no variable influence on nuclear compared to cytoplasmic expression of PR ($p < 0.001$) (Appendix D, Table D3). With continued hormonal influence (EPR), a comparative increase in nuclear PR expression ($p < 0.001$) was induced (Appendix D, Table D3), which was also noted visually (Figure 6B, D). With the combination of immunotherapy, a significant decrease in nuclear PR MFI ($p < 0.001$) compared to the cytoplasm was noted in the RU486+NK group (Appendix D, Table D1, D3), corresponding to qualitative analyses (Figure 3.7). With hormonal influences, the addition of NK cells to RU486-treated cells (EPR+NK) resulted in higher nuclear expression comparatively ($p < 0.001$) (Appendix D, Table D1, D4). This indicates that NK cell influence may cause MCF-7 cells to mediate PR translocation between cytoplasmic and nuclear compartments.

When RU486 treatment groups were analysed in relation to the baseline dDMSO control, it was found that RU486 alone induced a significant reduction in both nuclear and cytoplasmic PR expression (Figure 3.9). Conversely, the addition of a hormonal treatment (EPR) revealed increased PR MFI in both cellular compartments ($p < 0.001$) in comparison to both the diluent control (dDMSO), the hormone control (EP), and RU486 treatment alone (Figure 3.9). These results were difficult to correlate with qualitative observations (Figure 3.5, Figure 3.6). However, the quantitative results indicate that RU486 in a hormonal environment significantly increases PR expression, an action in direct opposition to that of Anastrozole treatment. Notably, the PR MFI was much higher in the nucleus in the EPR group compared to all other

treatment groups (Figure 3.9), which corresponded with visual observations of dense green fluorescent staining within the nucleus (Figure 3.6D).

Within the combined hormone-therapy and immunotherapy treatment, NK cell mediation of RU486 function (RU486+NK) was shown to reduce ($p<0.001$) PR nuclear and cytoplasmic expression compared to the diluent control (dDMSO) (Figure 3.10). This reduction was distinct and could be assessed visually (Figure 3.5B, Figure 3.7B). With additional hormones (EPR+NK), PR MFI showed a significant reduction in both cellular compartments when compared with the diluent control and the hormone control (EP) (Figure 3.10). Qualitatively, the EPR+NK treatment group had distinct nuclear and cytoplasmic PR localisation (Figure 3.7D), which appeared greatly reduced compared to the diluent control, and only marginally different to the hormone control (EP) (Figure 3.5). Notably, in comparison to RU486 alone, EPR+NK induced a significant decrease in cytoplasmic PR MFI, with the converse evident in the nucleus. This result echoes that seen in the effects of Anastrozole, with and without hormonal stimuli but under NK cell mediation, further indicating that NK cells may mediate the action of hormone-therapy drugs on PR expression.

We thereafter compared the effects of hormone-therapy alone to combined hormone-therapy and immunotherapy (Figure 3.11) on PR expression. It was noted that the addition of NK cells to the culture system, whether under hormonal influence or not (RU486+NK and EPR +NK) induced a significant decrease in PR MFI in both the nucleus and the cytoplasm (Figure 3.11), corresponding with visual observations (Figure 3.5 – Figure 3.7). These results reflect that while RU486 modulation of PR appears dependent on the hormonal environment, as evidenced by the large nuclear and cytoplasmic increase in PR expression in the hormone-therapy group, NK cell-mediation of cancer cells may allow RU486 to function independently of hormonal influences, as evidenced by the great reduction in PR expression.

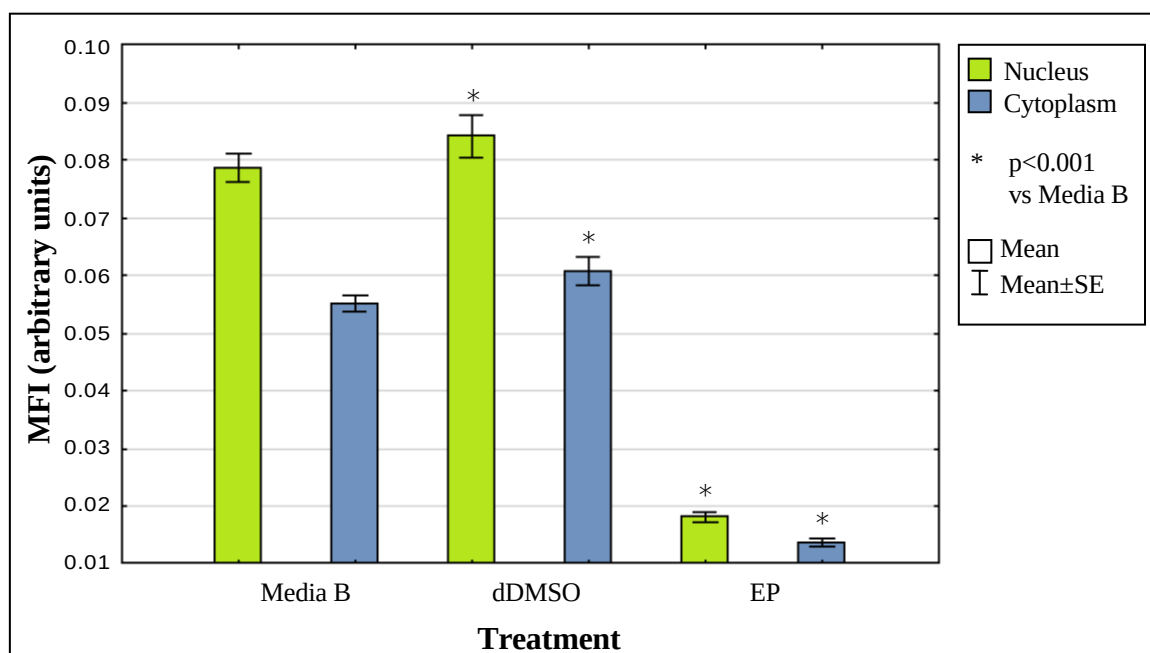


Figure 3.8. A bar graph showing the nuclear and cytoplasmic PR MFI for the control treatments

Nuclear expression of PR was consistently higher than cytoplasmic expression in all control treatment groups. Cells treated with diluent alone (dDMSO) exhibited a higher PR MFI in both the nucleus and cytoplasm ($p < 0.001$) compared to the Media B control. The hormone control (EP) induced a significant reduction in nuclear and cytoplasmic PR MFI ($p < 0.001$) compared to the Media B control. Given that all drugs and hormones were reconstituted in DMSO, the dDMSO control group was used as comparator group instead of the Media B control for all subsequent analyses.

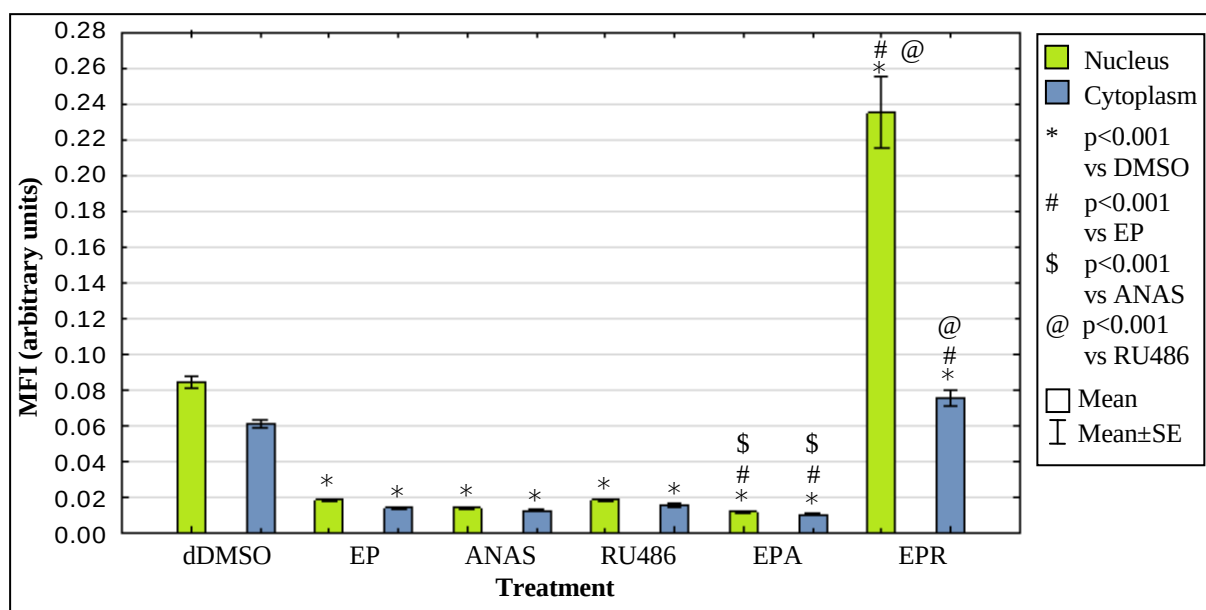


Figure 3.9. A bar graph showing the nuclear and cytoplasmic PR MFI for the control and hormone-therapy treatments

Treatment with EP, ANAS, RU486 and EPA induced a significant decrease ($p < 0.001$) in PR MFI in the nucleus and cytoplasm, whereas EPR induced a significant increase ($p < 0.001$) in both compartments as compared to the dDMSO control group. When compared to the hormone control (EP), the addition of hormones to Anastrozole-treated cells (EPA) induced a significant decrease in PR MFI and a conversely large increase in PR MFI in RU486-treated cells (EPR) in the nucleus and cytoplasm. The addition of hormones (EPA) resulted in a significant decrease in PR MFI compared to Anastrozole alone, and a significant increase in PR MFI compared to RU486 alone, in both cellular compartments. This indicates that hormones may differentially affect PR expression depending on the type of hormone-therapy used.

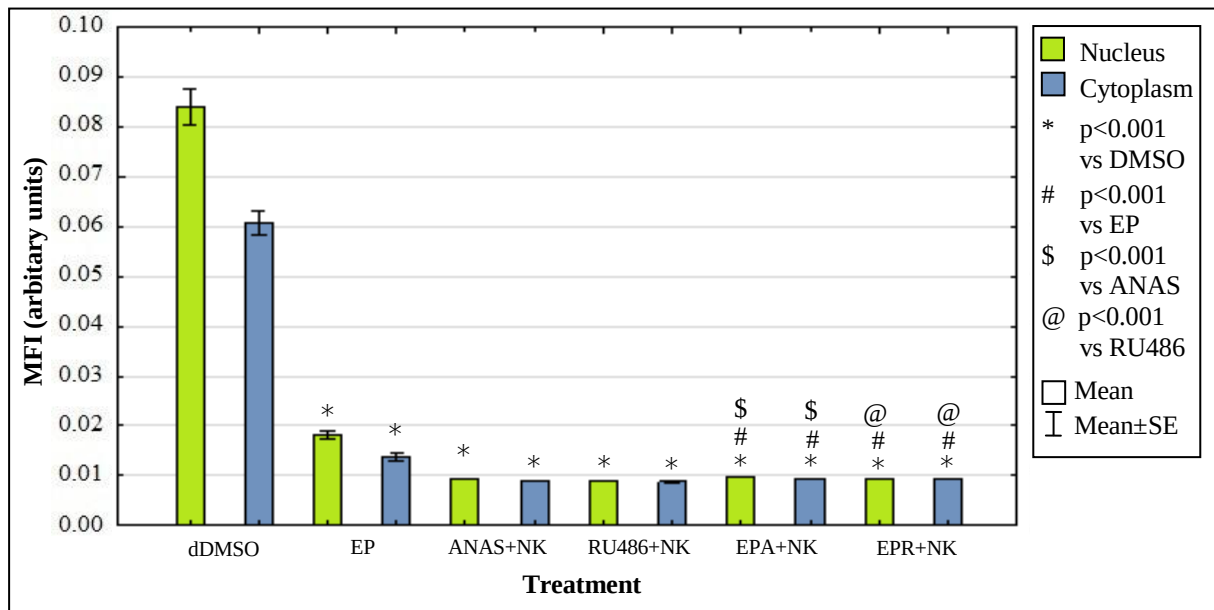


Figure 3.10. A bar graph showing the nuclear and cytoplasmic PR MFI for the control and combined hormone-therapy with immunotherapy treatments.

With combined hormone-therapy and immunotherapy, Anastrozole treatment (ANAS+NK) and RU486 treatment (RU486+NK) resulted in a significant decrease ($p < 0.001$) in PR expression in both nuclear and cytoplasmic compartments compared to the diluent control (dDMSO). With the addition of hormones to these treatment groups (EPA+NK, EPR+NK), PR expression remained significantly reduced ($p < 0.001$) compared to both the diluent control and hormone control (EP). However, compared to matched samples without hormonal stimuli (ANAS+NK, RU486+NK), the addition of hormones, further increased ($p < 0.001$) nuclear PR MFI and decreased cytoplasmic PR MFI ($p < 0.001$).

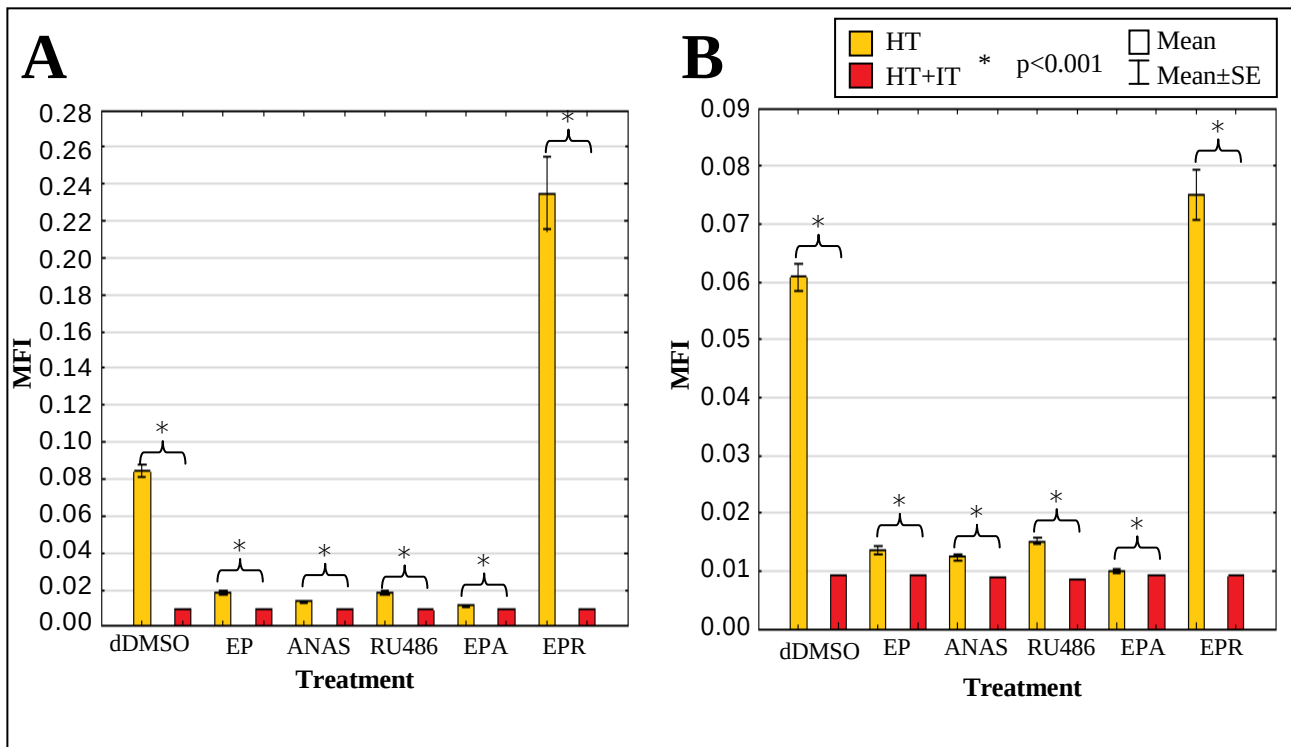


Figure 3.11. Bar graphs showing the cytoplasmic and nuclear PR MFI for the hormone-therapy compared to the combined hormone-therapy with immunotherapy treatments

A: Cytoplasmic PR MFI. **B:** Nuclear PR MFI. The addition of NK cells (HT+IT, red bars) resulted in a significant decrease in overall PR MFI ($p < 0.001$) for controls (dDMSO vs dDMSO+NK, EP vs EP+NK) and treatments, compared to hormone-therapy alone (HT, orange bars). The highest MFI occurs in EPR versus EPR+NK treatments, indicating that RU486 modulation of PR expression is greatly affected by the hormonal milieu and NK cell influence. This trend, albeit with lower MFI in both nuclear and cytoplasmic compartments, was noted for Anastrozole treatments as well.

The Effects of Hormone-Therapy and Combined Therapy on ER α Immunolocalisation

ER α is visible in both the cytoplasm and the nucleus of the MCF-7 cells in all control groups and treatment groups. The Media B control cells exhibited, in the majority of the cells, an intermediate ER α cytoplasmic fluorescent intensity with a few cells exhibiting a high cytoplasmic fluorescent intensity (Figure 3.12A). The EP control induced a very low cytoplasmic fluorescent intensity, almost indistinguishable, with putative nuclear ER α localisation as evinced by some light blue staining due to green fluorescence (Alexa Fluor 488) overlap with DAPI (Figure 3.12B). Conversely, cells in the DMSO diluent control show an intermediate to high cytoplasmic fluorescent intensity, primarily in the perinuclear region, with a high nuclear fluorescent intensity (Figure 3.12C).

ER α expression was noted in the cytoplasm and nucleus of all the hormone-therapy (HT) treatment groups (ANAS, RU486, EPA and EPR). Specifically, compared to the Media B and dDMSO controls (Figure 3.12A, C) an intermediate level of cytoplasmic fluorescence (green) was noted in cells treated with Anastrozole and RU486 (Figure 3.13A, B). It appeared, by the light blue nuclear stain, that the RU486-treated cells demonstrated a higher nuclear ER α localisation compared to all other HT treatments (Figure 3.13). Cells in this treatment group also displayed distinct perinuclear ER α presence. Compared to the hormone control (EP) (Figure 3.12B), cells treated with combined hormones and hormone-therapy drugs (EPA, EPR) showed marginally higher cytoplasmic fluorescent intensity (Figure 3.13C, D). However, in comparison to Anastrozole alone (ANAS), and RU486 alone (RU486) (Figure 13A, B), matched samples with hormonal treatment (EPA, EPR) showed lower cytoplasmic fluorescence. Nuclear localisation was difficult to determine overall in EPA and EPR treated cells, with only a few cells exhibiting a high nuclear fluorescent intensity (Figure 3.13C, D).

With NK cell co-culture, the combined hormone-therapy and immunotherapy (HT+IT) treatments (ANAS+NK, RU486+NK, EPA+NK and EPR+NK) induced an overall intermediate cytoplasmic fluorescent intensity. While again difficult to determine, cells in the ANAS+NK appeared to exhibit more nuclear ER α localisation (Figure 3.14A) compared to those cells in the RU486+NK group, which demonstrated higher cytoplasmic expression (Figure 3.14B). The addition of hormones resulted in a noticeable increase in cytoplasmic fluorescence in both the EPA+NK and EPR+NK treatment groups (Figure 3.14C, D) when compared to matched samples in the HT group alone (EPA and EPR) without NK influence (Figure 3.13C, D). This may indicate that under NK influence MCF-7 cells increase ER α expression.

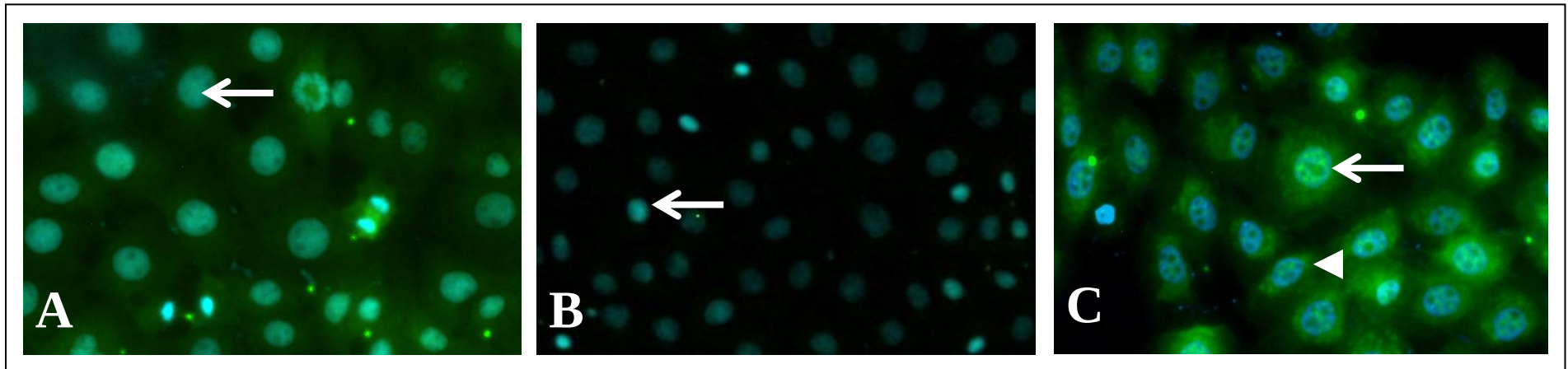


Figure 3.12. Representative photomicrographs showing the effects of control treatments on ER α expression in MCF-7 cells

Control treatment groups: **A:** Media B. **B:** EP. **C:** dDMSO. Fluorescent microscopy, 40x magnification. **A:** Media B treatment results in an intermediate cytoplasmic expression (green) with an increase in intensity in the nucleus (arrow, light blue). **B:** EP treated cells exhibited an overall very low ER α intensity expression with some cells displaying possible nuclear expression (arrow, light blue) **C:** The DMSO diluent control induced an intermediate cytoplasmic fluorescent intensity (arrowhead) with a high nuclear and perinuclear fluorescent intensity (arrow).

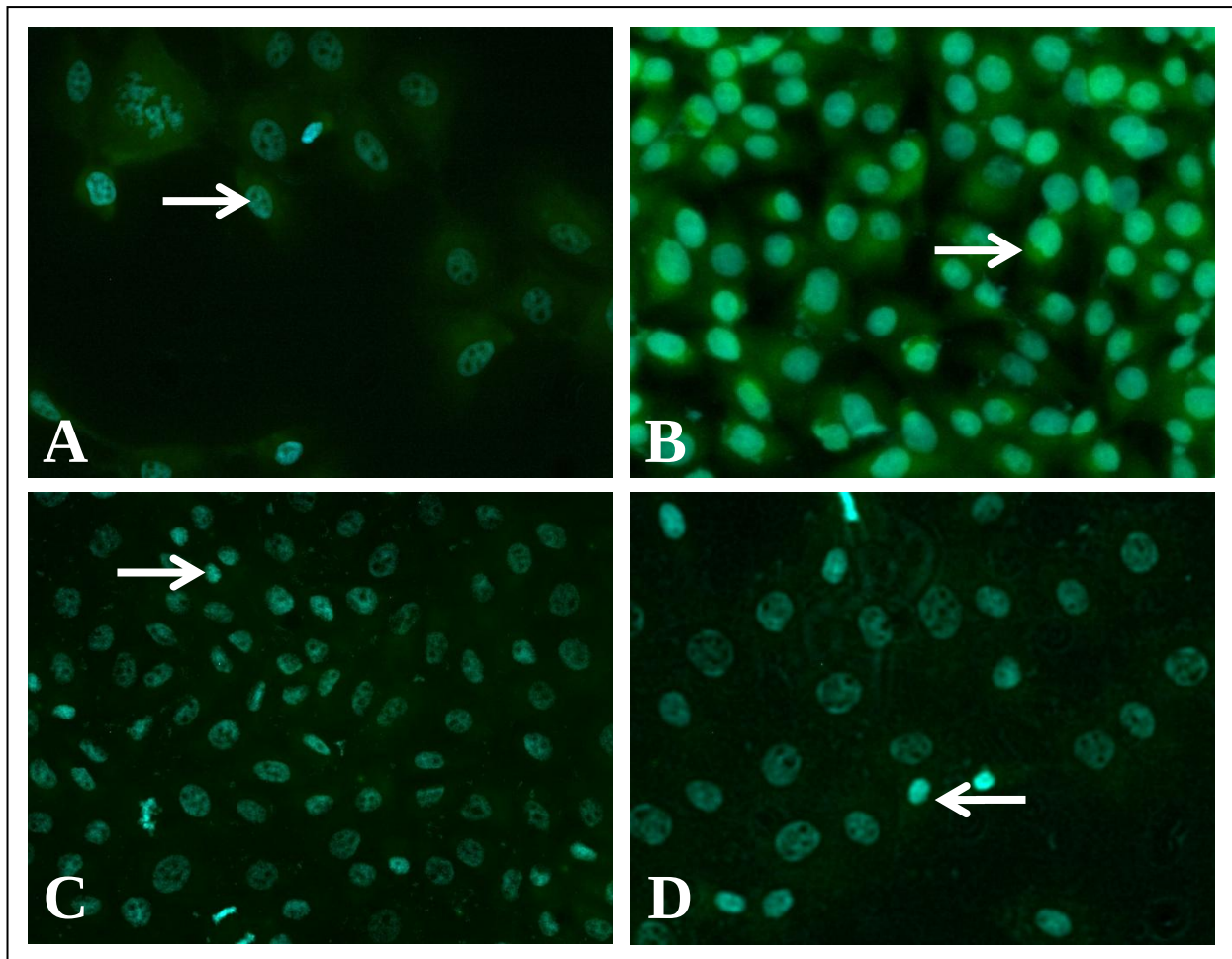


Figure 3.13. Representative photomicrographs showing the effects of hormone-therapy on ER α expression in MCF-7 cells

Treatment groups: **A:** ANAS **B:** RU486. Fluorescent microscopy, 20x magnification **C:** EPA **D:** EPR. Fluorescent microscopy, 40x magnification. **A:** The cells treated with Anastrozole showed intermediate levels of ER α localisation (green) in the cytoplasm. Some nuclei appear to show higher nuclear fluorescent intensity (arrow, light blue)). **B:** RU486 treatment induced a high fluorescent intensity in the perinuclear region (arrow) and cytoplasm. Light blue nuclei indicated a preponderance of ER α expression. **C:** Cells in the EPA group show low cytoplasmic ER α fluorescent intensity, with few cells demonstrating higher nuclear fluorescent intensity (arrow). **D:** The EPR group exhibited a low cytoplasmic fluorescent intensity with few cells showing a high nuclear fluorescent intensity (arrow).

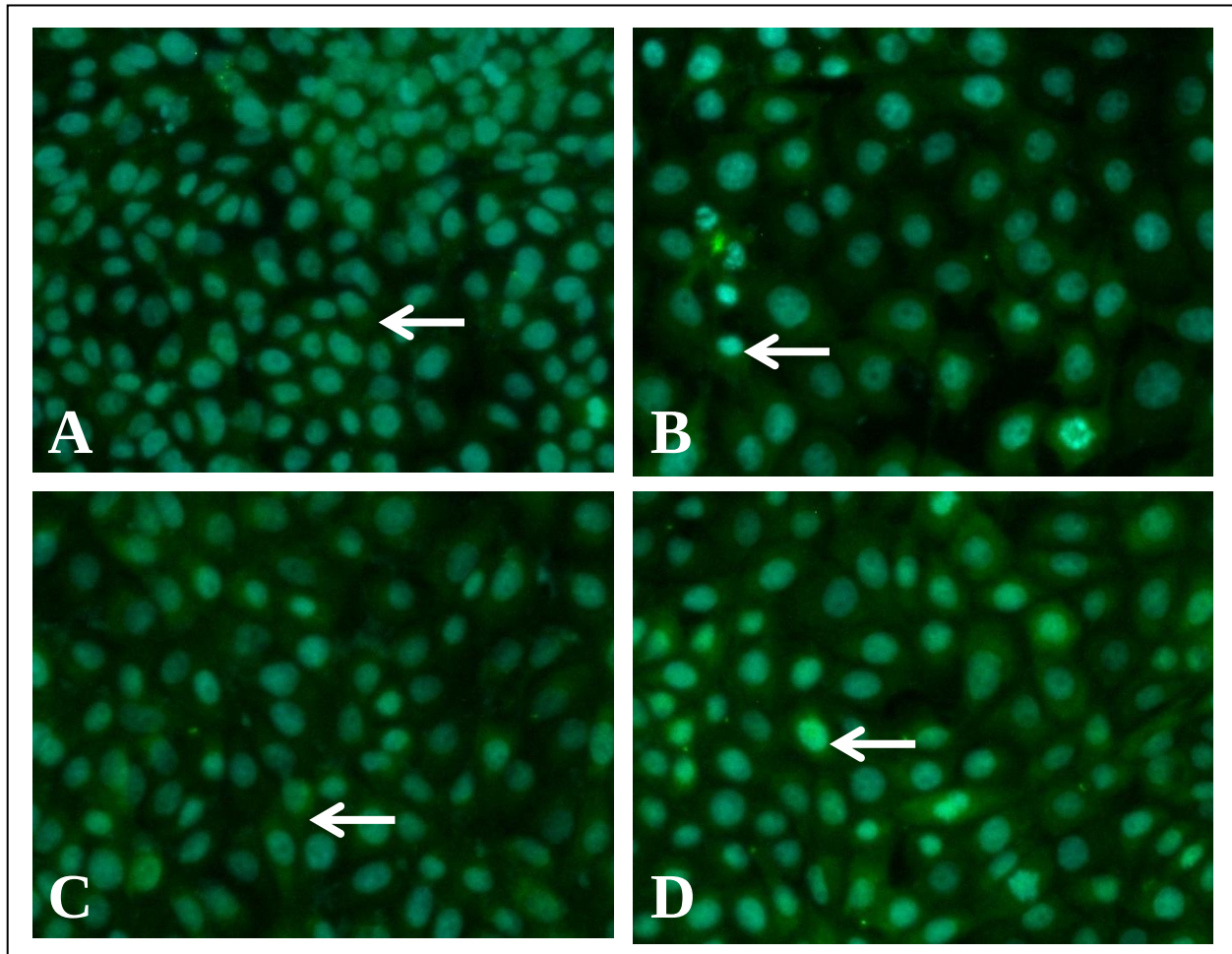


Figure 3.14. Representative photomicrographs showing the effects of combined hormone-therapy and immunotherapy on ER α expression in MCF-7 cells

Treatment groups: **A:** ANAS+NK **B:** RU486+NK **C:** EPA+NK **D:** EPR+NK. Fluorescent microscopy, 20x magnification (digitally magnified a quarter of the image). **A:** The ANAS+NK treated cells shows a majority displaying an intermediate ER α cytoplasmic fluorescent intensity (arrow, green). **B:** Cells in the RU486+NK group exhibited a higher cytoplasmic fluorescent intensity with few cells showing high nuclear ER α expression (arrow, light blue). **C:** Cells in the EPA+NK treatment group showed a higher fluorescent intensity in the cytoplasm (arrow). **D:** Cells in the EPR+NK group showed a high nuclear fluorescent intensity in the majority of the cells (arrow, light blue), and a high cytoplasmic fluorescent intensity.

Images were thereafter analysed quantitatively using CellProfiler. The normality tests Shapiro-Wilk, Jarque-Bera and χ^2 were performed on the data produced by CellProfiler, with significance set at $p=0.001$. These include total cellular expression, nuclear expression and cytoplasmic expression. Most of the data sets were not normally distributed ($p<0.001$) (see Appendix D, Table D8) thus the non-parametric Kruskal-Wallis one-way analysis of variance was used to compare each data set and pairwise comparisons were performed between and within the data sets with the Mann-Whitney test, with significance for both set at $p=0.001$ (Appendix D, Tables D9-D12).

Control treatments were first assessed to investigate whether ER α expression varied. Compared to the Media B control, the DMSO diluent control (dDMSO) and hormone control (EP) induced a significant decrease ($p<0.001$) in ER α mean fluorescent intensity (MFI) in both the nucleus and cytoplasm (Figure 3.15). While qualitative analysis confirmed this reduction in the EP group, this was not readily discernible in the dDMSO group (Figure 3.12). Importantly, this indicates that the diluent DMSO has an effect on ER α expression. As the hormones and drugs were all dissolved in DMSO, to isolate their effects from that of the DMSO, treatments (including the EP hormone control) were compared to the dDMSO control instead of the Media B control. In both areas of the cell the EP hormone control induced a significantly decreased ($p<0.001$) ER α MFI compared to that of the dDMSO control (Appendix D, Tables D7, D9). This result was also observed visually (Figure 3.12). The EP control group remained as an additional hormone control group for combined hormone and drug treatments (EPA and EPR). Furthermore, in addition to these controls when comparing the effects of hormone therapy alone against hormone-therapy and immunotherapy, the additional control comparator groups, dDMSO+NK and EP+NK, were also employed.

Across the control groups (Media B, dDMSO and EP) there was a significant increase ($p<0.001$) in ER α mean fluorescent intensity (MFI) in the nucleus as compared to the cytoplasm (Appendix D, Tables D7, D9). While a lighter blue nucleus is associated with ER α localisation, due to the overlap between the green Alexa Fluor 488 and the blue DAPI stain, nuclear localisation was difficult to assess with a degree of accuracy qualitatively (Figure 3.12).

The Effects of Anastrozole on ER α MFI

We firstly determined whether ER α expression varied between nuclear and cytoplasmic compartments within treatment groups. It was found that nuclear fluorescent intensity was significantly higher ($p < 0.001$) compared to the cytoplasmic compartment whether in all hormone-therapy treatment groups whether under immune-mediation or not (Appendix D, Tables D7, D9, D10). This was difficult to discern visually (Figure 3.13, Figure 3.14).

The effects of hormone-therapy treatments on ER α expression were thereafter investigated. When compared to the diluent DMSO control, Anastrozole treatment induced a significant increase ($p < 0.001$) in cytoplasmic ER α MFI and a conversely significant decrease ($p < 0.001$) in nuclear MFI (Figure 3.16). With the addition of hormones (EPA), compared to the hormone control (EP) there was a significant increase in ER α MFI (Figure 3.16). However, when this group (EPA) was compared to the diluent control, and more importantly to Anastrozole treatment alone (ANAS), there was a significant decrease in ER α MFI in both cellular compartments (Figure 3.16). Visually, this was discernible particularly with regard to the cytoplasm (Figures 3.12, 3.13). The results indicate in a hormonal environment, Anastrozole treatment reduces overall ER α expression.

When NK cells were added (combined hormone-therapy and immunotherapy treatments), Anastrozole with hormones (EPA+NK) and without (ANAS+NK) significantly decreased ($p < 0.001$) ER α MFI in the nuclear compartment compared to the dDMSO control (Figure 3.17). However, the aforementioned groups also showed a significant increase ($p < 0.001$) in the cytoplasmic compartment compared to the diluent control. When compared to the hormone control, the EPA+NK group evidenced a significant increase in both nuclear and cytoplasmic ER α MFI (Figure 3.17). This was mimicked in comparison with the effects of Anastrozole alone under NK mediation (ANAS+NK). While qualitative analysis bears out these results, it remains that nuclear localisation was difficult to assess visually (Figure 3.12, Figure 3.14) with more accurate results emanating from the quantitative assessment. Furthermore, quantitative assessment shows that NK cell mediation under hormonal influence, results in Anastrozole effectively inducing an increase in ER α expression.

Comparing the effectiveness of hormone-therapy to combined hormone-therapy and immunotherapy (Figure 3.18), it was noted that immune mediation significantly reduced ($p < 0.001$) the overall ER α MFI in the dDMSO control (dDMSO vs dDMSO+NK), and

Anastrozole group (ANAS vs ANAS+NK). Conversely, NK cell presence resulted in a significant increase ($p<0.001$) in overall ER α MFI in the hormone control group (EP vs EP+NK), which was mimicked in the EPA group as well. These results indicate that NK cells mediate the expression of ER α by MCF-7 cells depending on the hormonal environment.

The Effects of RU486 on ER α MFI

Assessment of ER α expression in the nuclear and cytoplasmic compartments, within treatment groups was first conducted. Within the RU486 alone treatment group (RU486), no significant difference ($p>0.001$) was found between ER α in the nucleus and cytoplasm (Appendix D, Tables D7, D9). However, under continued hormonal influence (EPR) and under NK cell mediation (RU486+NK, EPR+NK) ER α expression significantly increased in the nucleus as opposed to the cytoplasm (Appendix D, Tables D7, and D10). Qualitatively, the reduction in cytoplasmic ER α expression was noted in the EPR group (Figure 3.13D); however, differences between the cytoplasmic and nuclear expression of ER α were more difficult to discern accurately in the RU486+NK and EPR+NK groups (Figure 3.14B, D).

When the effects of hormone-therapy treatments were assessed, it was found that compared to the dDMSO control, RU486 treatment induced a significant increase ($p<0.001$) in ER α MFI in the cytoplasm; however, there was no significant difference detected in the nucleus ($p>0.001$) (Figure 3.16). With the addition of hormones (EPR) there was a significant decrease in ER α MFI when compared to the diluent control, and RU486 treatment alone (Figure 3.16). However, when compared to the hormone EP control, the EPR group showed a significant increase ($p<0.001$) in ER α MFI. These results were comparable with qualitative observations with marked reduction in both cellular compartments compared to RU486 alone (Figure 3.12C, Figure 3.13B, D). This indicates that the addition of hormones affects RU486 modulation of ER α expression, specifically effecting a decrease in expression compared to RU486 alone, comparable to the results seen with Anastrozole treatment.

The combination of the RU486 hormone-therapy with NK cell immunotherapy showed a significant decrease ($p<0.001$) in nuclear ER α MFI and significant increase ($p<0.001$) in cytoplasmic MFI when compared to the dDMSO control (Figure 3.17). These results mimicked the qualitative observations (Figure 3.12C, Figure 3.14B). With the addition of hormones, RU486 action resulted in a significant increase ($p<0.001$) in MFI compared to both the EP hormone control and RU486 alone (RU486+NK), in both the nuclear and cytoplasmic

compartments (Figure 3.17). The results indicate that in a hormonal environment under the influence of NK cell mediation, RU486 action results in greater expression of ER α . This result echoes that of Anastrozole treatment under NK cell and hormonal mediation.

We further compared the effects of hormone-therapy and combined hormone-therapy and immunotherapy on ER α expression. It was noted that NK cell mediation significantly reduced ER α MFI in the dDMSO control (dDMSO vs dDMSO+NK) (Figure 3.18). Interestingly the reverse was identified in the EP control group with NK cell incubation inducing an increase in ER α MFI (Figure 3.18). RU486 alone induced the highest ER α expression in both nuclear and cytoplasmic compartments. However, with the addition of immunotherapy (RU486+NK), expression in both compartments was significantly reduced ($p < 0.001$) (Figure 3.18). Conversely, with the addition of hormones (EPR), immunotherapy (EPR+NK) induced a significant increase in ER α MFI. These results indicate that RU486 functions in modulation of ER α and that this modulation may be affected by hormonal and immune influences. Specifically the addition of NK cells, ameliorates the reduction of ER α expression by the addition of hormones to RU486 treatment.

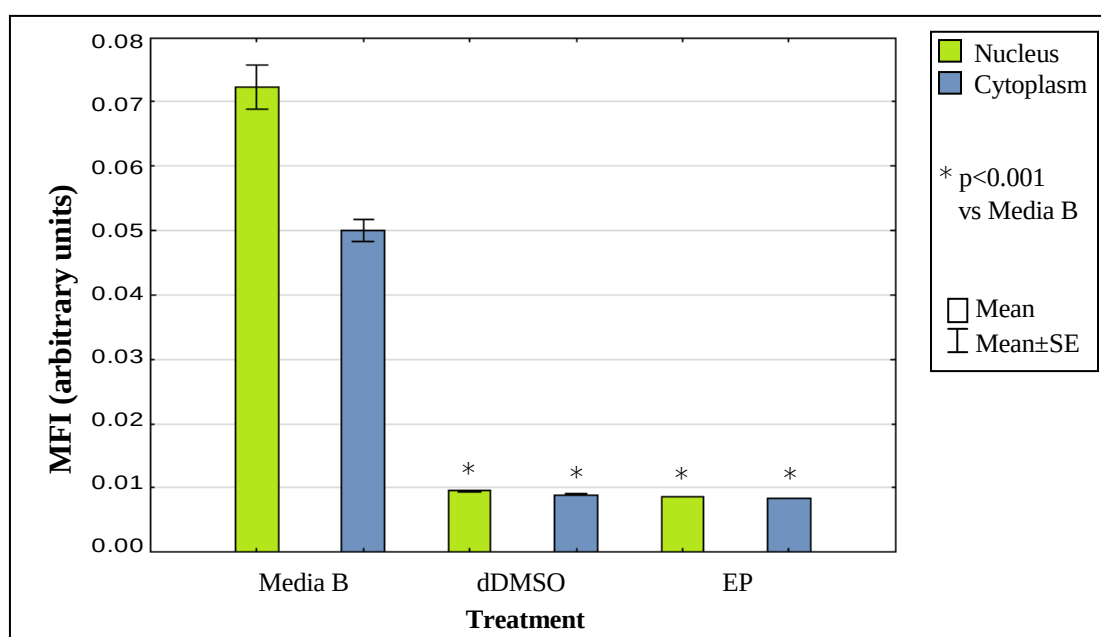


Figure 3.15. A bar graph showing the nuclear and cytoplasmic ER α MFI for the control treatments

In all control groups nuclear ER α expression was higher than that of the cytoplasm. The dDMSO control and EP control showed a significantly decreased ER α MFI ($p < 0.001$) compared to the Media B control for both the nucleus and the cytoplasm. Since all drugs and hormones were reconstituted in DMSO, the dDMSO control group was used as comparator group instead of the Media B control for all subsequent analyses.

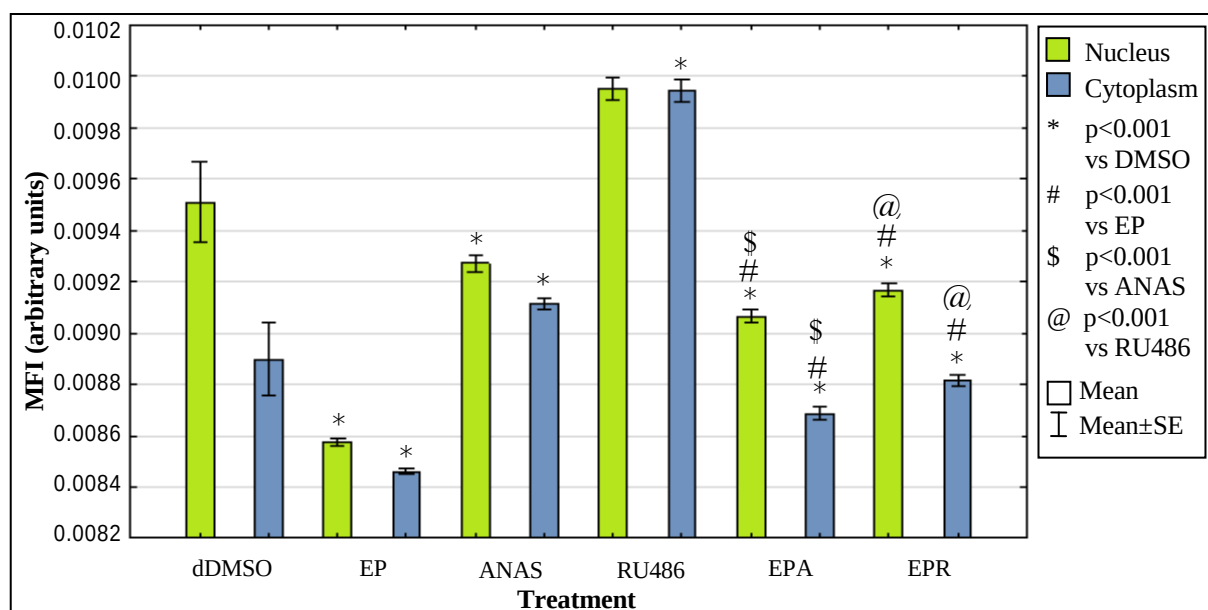


Figure 3.16. A bar graph showing the nuclear and cytoplasmic ER α MFI for the control and hormone-therapy treatments

Anastrozole treatment induced a significant decrease ($p < 0.0001$) in nuclear ER α MFI and a converse significant increase in cytoplasmic ER α MFI compared to the diluent control (dDMSO). RU486-treated cells exhibited a higher nuclear ER α MFI with only cytoplasmic expression being significantly higher to that of the diluent control. Under hormonal stimulation, Anastrozole-induced (EPA) and RU486-induced (EPR) ER α MFI nuclear and cytoplasmic intensity was significantly increased compared to the hormone control (EP); however, ER MFI in both groups was decreased ($p < 0.001$) compared to the diluent control and each hormone-therapy drug alone (ANAS, RU486) in both cellular compartments. This indicates that the presence of hormones modulate the function of Anastrozole and RU486 in mediation of ER α expression.

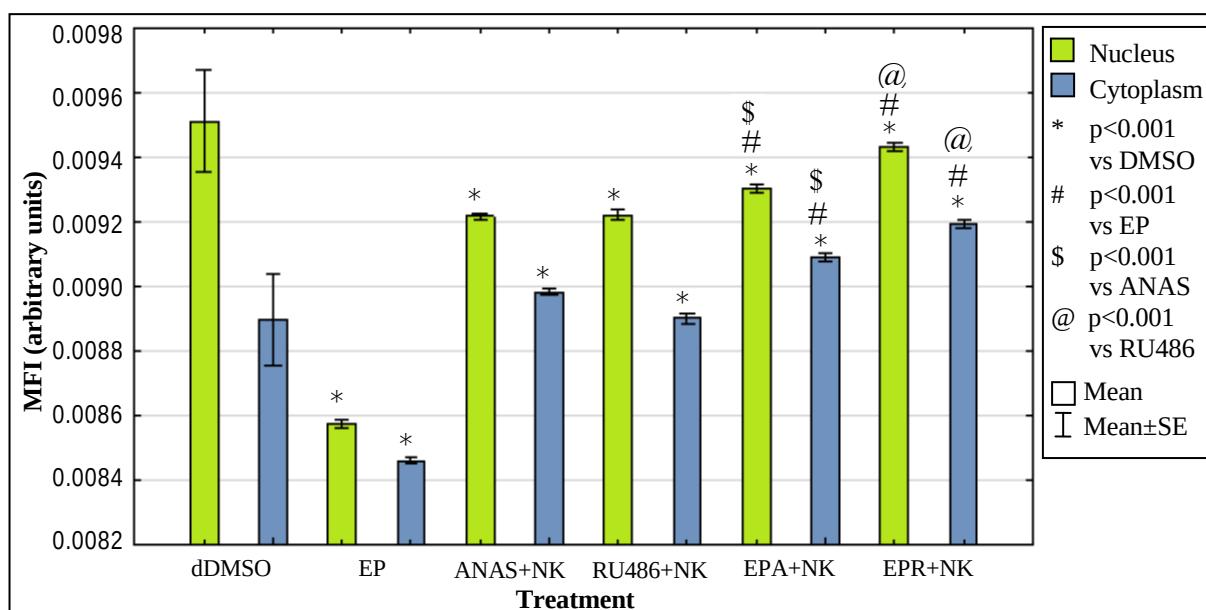


Figure 3.17. A bar graph showing the nuclear and cytoplasmic ER α MFI for the control and combined hormone-therapy with immunotherapy treatments

Under immune-mediation, Anastrozole and RU486 induced a significant decrease in nuclear ER α and a significant increase in cytoplasmic ER α whether alone (ANAS+NK, RU486+NK) or with hormonal influence (EPA+NK, EPR+NK) compared to the diluent control (dDMSO). However, EPA and EPR caused a significant increase in MFI in both the cytoplasm and the nucleus when compared to the EP hormone control, and compared to matched samples that were not under hormonal influence (ANAS+NK, RU486+NK). This indicates that the action of hormone-therapy drugs may be modulated by the presence of hormones.

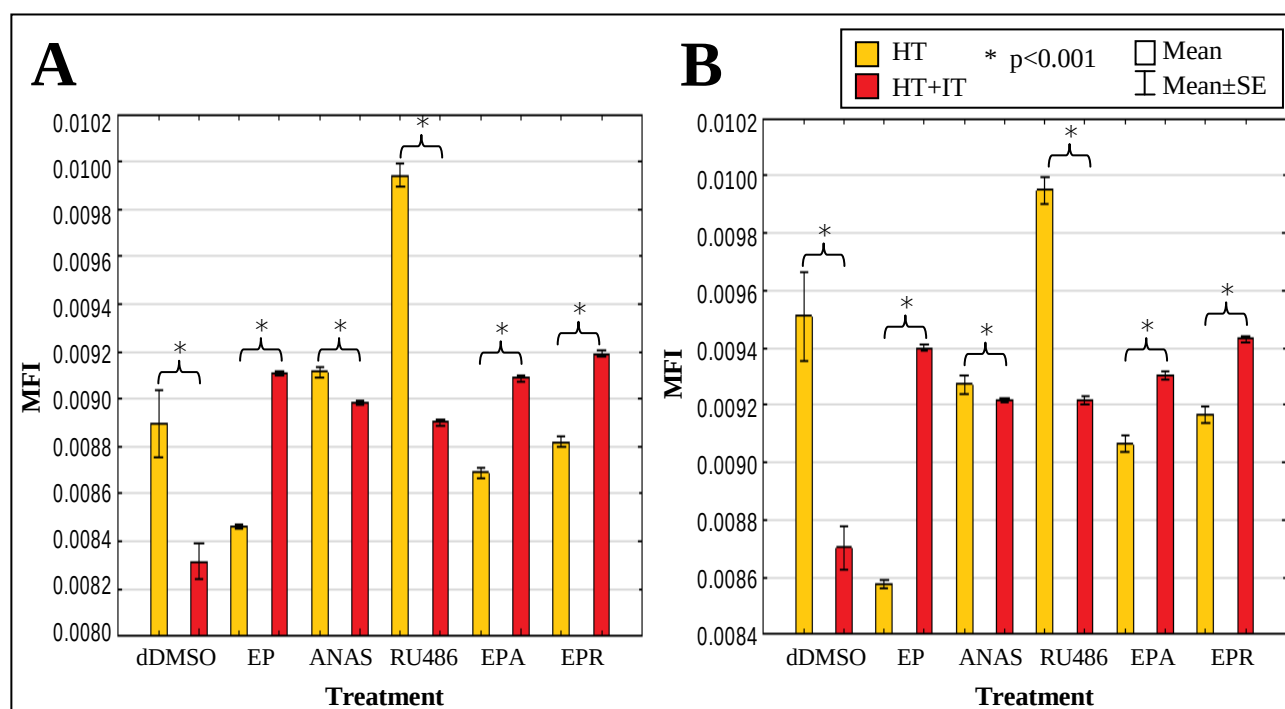


Figure 3.18. Bar graphs showing the cytoplasmic and nuclear ER α MFI for the hormone-therapy compared to the combined hormone-therapy with immunotherapy treatments

A: Cytoplasm. B: Nucleus. In cells treated with the diluent DMSO, the addition of NK cell immunotherapy (HT+IT, red bars) induced a significant reduction ($p < 0.001$) in ER α expression in both the nucleus and cytoplasm compared to hormone-therapy alone (HT, orange bars). Conversely in the hormone control (EP), NK cell mediation resulted in a significantly higher ER α MFI in both cellular compartments. For cells treated with Anastrozole and RU486 alone, immunotherapy showed a significant reduction ($p < 0.001$) in fluorescence intensity in both the nucleus and the cytoplasm. However with the addition of hormones (EPA, EPR), the effects of the drugs was modulated and immunotherapy resulted in a significant increase in nuclear and cytoplasmic ER α MFI instead.

The Effects of Hormone-Therapy and Combined Therapy on MUC1 Immunolocalisation

For all controls and treatments performed on the MCF-7 cells, it was observed that there were more polygonal cells than mesenchymal cells. Both the mesenchymal cells and the polygonal cells vary in MUC1 intensity. MUC1 is visible in both the cytoplasm (red fluorescence) and the nucleus of the cells in all control groups and treatment groups (Figure 3.19). MUC1 nuclear localisation is described as red/purple staining due to the overlap of Alexa Fluor 594 (red) with the blue DAPI nuclear stain. Cells in the Media B control, which was employed for cell cycle synchronisation, exhibited intermediate cytoplasmic fluorescent intensity with areas of high perinuclear intensity. MUC1 was noticeably localised to the nucleus, as seen by the more reddish purple stained nuclei, with areas of red spots (Figure 3.19A). Compared to the Media B control, the EP hormone control treatment induced a lower level of MUC1 fluorescence. Perinuclear staining was evident, with nuclei demonstrating regions of MUC1 staining (red/purple spots within the blue-stained nucleus) (Figure 3.19B). Conversely, the cells in the dDMSO control group showed an intermediate cytoplasmic MUC1 fluorescent intensity with prominent perinuclear staining. Nuclear MUC1 was also noted (Figure 3.19C).

In the hormone-therapy (HT) groups (ANAS, RU486, EPA and EPR) MUC1 expression varied in intensity and localisation depending on treatment. Anastrozole and RU486 treated cells exhibited low to intermediate MUC1 cytoplasmic fluorescent intensity with a higher intensity in the perinuclear area and nucleus (Figure 3.20A, B). Cells treated with EPA show high cytoplasmic and nuclear fluorescent intensity (Figure 3.20C). EPR treated cells exhibited low to intermediate cytoplasmic intensity with an increased intensity in the perinuclear region (Figure 3.20D). The addition of hormones appeared to increase the intensity of MUC1 in comparison to use of hormone-therapy drugs alone.

In the hormone-therapy and immunotherapy (HT+IT) groups (ANAS+NK, RU486+NK, EPA+NK and EPR+NK) cytoplasmic MUC1 intensity increased (Figure 3.21) compared to hormone-therapy alone (Figure 3.20). Cells in the ANAS+NK, RU486+NK and EPR treatment groups exhibited intermediate to high MUC1 fluorescence in both the nucleus and cytoplasm (Figure 3.21A, B, D). The EPA+NK group induced the highest cytoplasmic fluorescent intensity with an increased intensity in the perinuclear and nuclear regions (Figure 3.21C)

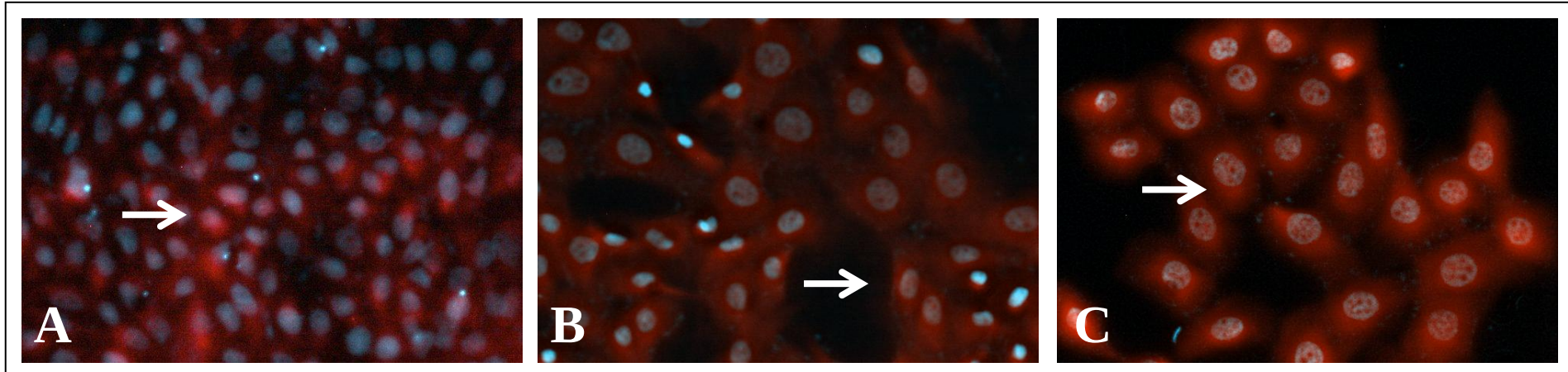


Figure 3.19. Representative photomicrographs showing the effects of control treatments on MUC1 expression in MCF-7 cells

Control treatment groups: **A:** Media B. **B:** EP. **C:** dDMSO. Fluorescent microscopy, 40x magnification. **A:** Media B treatment results in intermediate MUC1 cytoplasmic fluorescent intensity (red fluorescence) with the majority of cells reddish/purple stained nuclei indicative of MUC1 localisation (arrow, red/purple spots on blue DAPI-stained nuclei) and perinuclear fluorescent intensity (bright red fluorescence). **B:** EP treated cells exhibit overall intermediate MUC1 cytoplasmic fluorescent intensity with an increased intensity in the perinuclear region (arrow). **C:** The DMSO diluent control induced an intermediate cytoplasmic fluorescent intensity with high intensity MUC1 in the perinuclear region (arrow). Nuclear MUC1 is seen as red spots with the blue-stained nuclei.

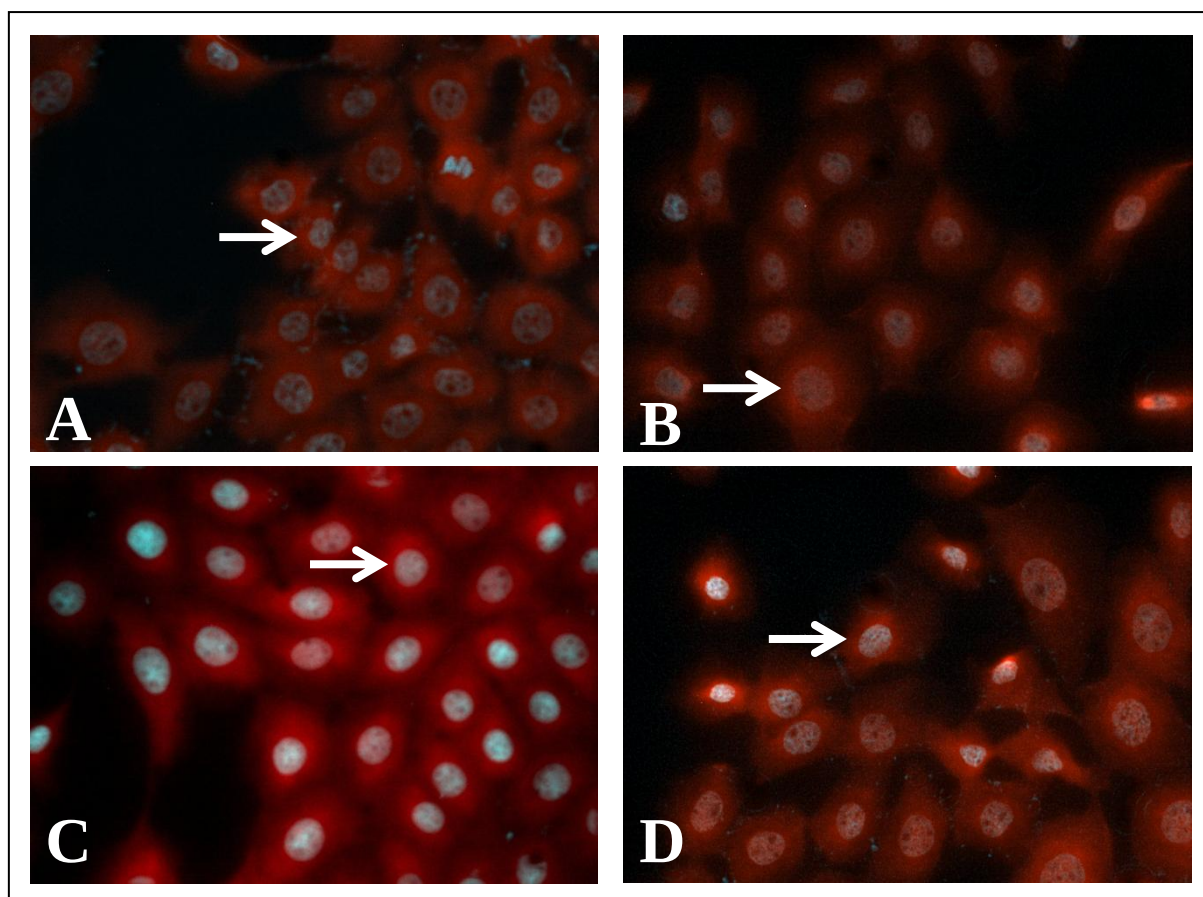


Figure 3.20. Representative photomicrographs showing the effects of hormone-therapy on MUC1 expression in MCF-7 cells

Treatment groups: **A:** ANAS **B:** RU486 **C:** EPA **D:** EPR. Fluorescent microscopy, 20x magnification. **A:** The cells treated with Anastrozole exhibited low to intermediate MUC1 cytoplasmic fluorescent intensity (red) with higher perinuclear intensity (arrow, bright red) and defined nuclear fluorescence (red spots within the purple-stained nucleus). **B:** The group treated with RU486 induced a low to intermediate cytoplasmic fluorescent intensity with an additionally increased perinuclear (arrow) and nuclear fluorescent intensity. **C:** EPA treated cells show a high cytoplasmic MUC1 fluorescent intensity, with a higher perinuclear fluorescent intensity (arrow). MUC1 localisation was high in the nucleus (reddish/purple nuclei). **D:** The EPR group induced a low to intermediate cytoplasmic intensity with an increased perinuclear (arrow) and nuclear intensity.

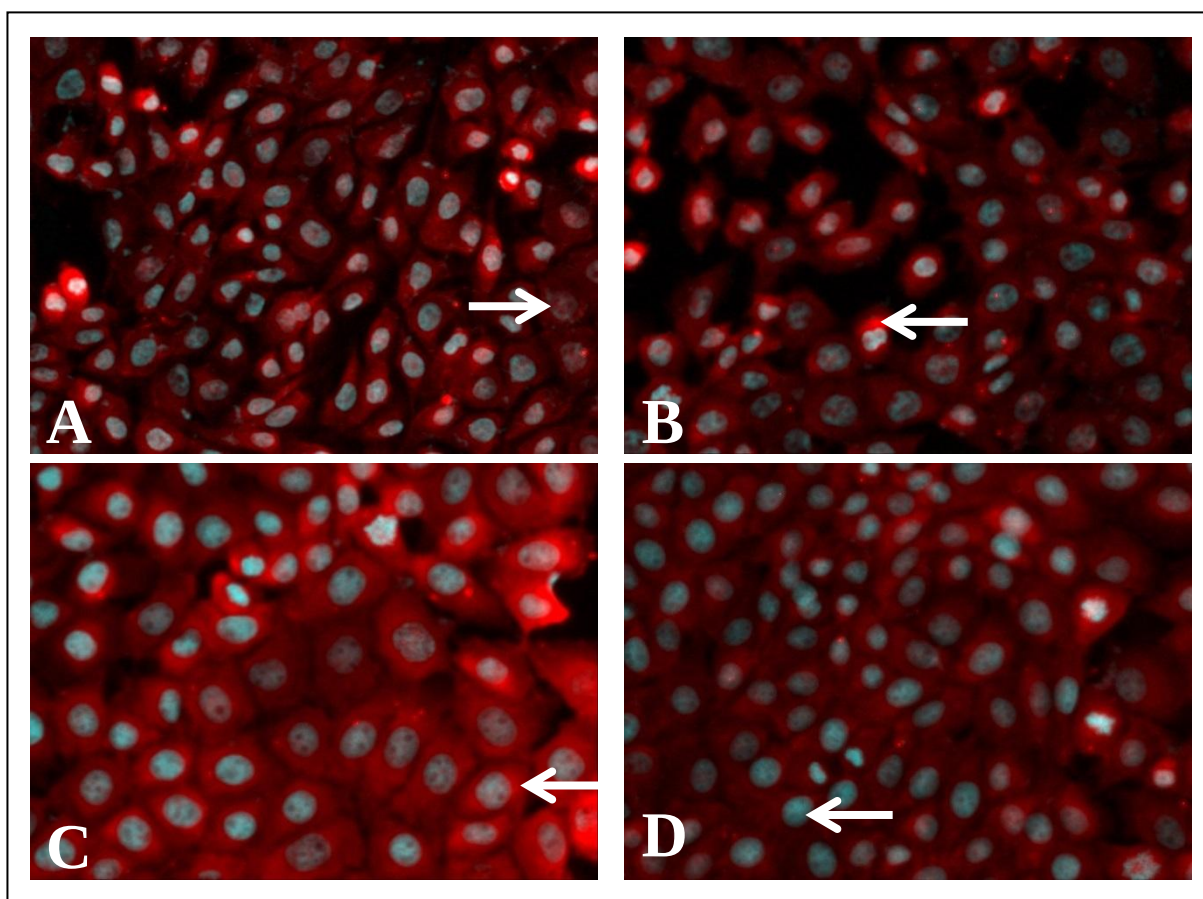


Figure 3.21. Representative photomicrographs showing the effects of combined hormone-therapy and immunotherapy on MUC1 expression in MCF-7 cells

Treatment groups: **A:** ANAS+NK **B:** RU486+NK **C:** EPA+NK **D:** EPR+NK. Fluorescent microscopy, 20x magnification (zoomed in on a quarter of the image). **A:** The ANAS+NK treated cells showed intermediate to high MUC1 cytoplasmic intensity (red) with a comparatively higher nuclear fluorescent intensity (arrow, red spots in blue-stained nucleus). **B:** RU486+NK treatment induced intermediate to high MUC1 cytoplasmic fluorescent intensity with notable nuclear localisation. Some cells exhibited distinct perinuclear fluorescent intensity (arrow, bright red). **C:** The EPA+NK group induced the highest level of MUC1 cytoplasmic fluorescence, with MUC1 evident in the nucleus (red spots) and increased nuclear, and perinuclear intensity (arrow). **D:** Cells treated with EPR+NK exhibit an intermediate to high cytoplasmic fluorescent intensity with a high nuclear fluorescent intensity in the majority of the cells (arrow).

Quantitative data was acquired from images using CellProfiler. The normality tests Shapiro-Wilk, Jarque-Bera and χ^2 were performed on the data with significance set at $p=0.05$. These include overall, nuclear and cytoplasmic expression (Appendix D, Table D13). Most of the data sets were not normally distributed ($p<0.05$) (Appendix D, Table D14) thus the non-parametric Kruskal-Wallis one-way analysis of variance was used to compare each data set and pairwise comparisons were performed between and within the data sets with the Mann-Whitney test, with significance for both set at $p=0.001$ (Appendix D, Table D15-18).

We first investigated whether MUC1 expression varied with control treatments. Compared to the Media B control, the DMSO diluent control (dDMSO) and the hormone control (EP) induced a significant decrease ($p<0.001$) in MUC1 mean fluorescent intensity (MFI) in the nucleus and the cytoplasm (Figure 3.22), which while noted in the qualitative assessment (Figure 3.19), was not discernible with a degree of accuracy visually particularly in the nucleus. Notably this result indicates that DMSO has an effect on MUC1 expression, as was noted for PR and ER α expression. Since the hormones and drugs were all dissolved in DMSO, to isolate their effects from that of the DMSO, for all ensuing analyses treatments were compared to the dDMSO control instead of the Media B control. The EP control was retained to determine the effects of the addition of hormones to the hormone-therapy treatments. Furthermore, where hormone-therapy (HT) was compared with combined hormone-therapy and immunotherapy (HT+IT), two additional controls were employed, dDMSO+NK and EP+NK.

The effects of all treatments on the MUC1 nuclear and cytoplasmic expression was first assessed. For all controls and treatments, with the exception of RU486 in the hormone-therapy group, there was a significant increase ($p<0.001$) in mean fluorescent intensity (MFI) in the nucleus as compared to the cytoplasm (Appendix D, Tables D13, D15, D16). Visually this was not readily apparent despite nuclei being consistently reddish-purple, with prominent red spots indicating MUC1 localisation (Figure 3.19 - Figure 3.21). Quantitative results have shed more light on MUC1 compartmentalisation than was readily visualised.

The Effects of Anastrozole on MUC1 MFI

Within the hormone-therapy treatment groups, Anastrozole treatment induced a significant increase ($p < 0.001$) in MUC1 MFI compared to the dDMSO control in both nuclear and cytoplasmic compartments (Figure 3.23). However, this was not readily discernible qualitatively (Figures 3.19, 3.20). The addition of hormones (EPA) resulted in a significant decrease ($p < 0.001$) in MUC1 expression in both cellular compartments when compared to the dDMSO control and Anastrozole treatment only (ANAS). However, compared to the hormone control (EP), EPA induced a small, yet significant decrease ($p < 0.001$) in cytoplasmic MUC1 while marginally increasing ($p < 0.001$) nuclear expression (Figure 3.23). This directly contradicts the visual assessment of MUC1 which appeared to exhibit high nuclear and cytoplasmic fluorescence, more so than either the diluent or the hormone control (Figure 3.19, Figure 3.20). However, given the large data set obtained quantitatively, it is suggested that the quantitative assessment is more reliable than subjective visual assessment. Furthermore, it is clear that under hormonal stimuli, the action of Anastrozole in increasing MUC1 expression is severely inhibited.

With the addition of NK cells, treatment with Anastrozole (ANAS+NK) or with hormones (EPA+NK) induced a significant decrease ($p < 0.001$) in MUC1 cytoplasmic and nuclear MFI compared to the dDMSO control (Figure 3.24). The addition of hormones (EPA+NK) resulted in significantly higher cytoplasmic and nuclear fluorescence compared to Anastrozole (ANAS+NK) and the EP hormone control (Figure 3.24). This was evident in qualitative observations (Figures 3.19, 3.21). These results indicate that MUC1 expression may be mediated by NK cells as well as the hormonal milieu.

To further investigate the results, we compared hormone-therapy with combined hormone-therapy and immunotherapy (Figure 3.25). It was noted that NK cell mediation (HT+IT), resulted in a decrease in MUC1 MFI in the diluent control groups (dDMSO vs dDMSO+NK), when compared with hormone-therapy alone (HT). This decrease while not significant within the cytoplasmic compartment, was significant ($p < 0.001$) when assessing the nuclear compartment. Combined hormone-therapy and immunotherapy, conversely, resulted in a significant increase in MUC1 expression in the both cellular compartments in the hormone control groups (EP vs EP+NK). By far the most drastic decrease ($p < 0.001$) in nuclear and cytoplasmic MUC1 MFI observed under NK cell mediation was that in the Anastrozole treatment group. As seen previously, the addition of hormones to Anastrozole treatment (EPA)

reduced MUC1 MFI. However, compared with hormone-therapy alone (EPA), a significant increase in MUC1 expression in both cellular compartments was noted with combined immunotherapy (EPA+NK). The results thus indicate that in a hormonal environment, the induction effects of Anastrozole on MUC1 are attenuated. Furthermore, NK cell presence may cause MCF-7 cells to alter the expression of this biomarker, inducing an increase in MUC1 expression.

The Effects of RU486 on MUC1 MFI

Within the hormone-therapy treatment groups, RU486 treatment induced a significant decrease ($p < 0.001$) in only nuclear MUC1 MFI as compared to the dDMSO control (Figure 3.23), complimenting visual observations (Figure 3.19, 3.20). With the addition of hormones (EPR), a significant reduction ($p < 0.001$) in both cytoplasmic and nuclear MUC1 expression was found compared to the dDMSO control (Figure 3.23). However, when compared to the hormone control (EP) and RU486 treatment alone (RU486), EPR induced a small, yet significant ($p < 0.001$) decrease in MUC1 MFI in the cytoplasmic compartment and increase in the nuclear compartment (Figure 3.23). Qualitative observations are only tentatively linked to these results, and no definitive association can be made with a degree of accuracy (Figures 3.19, 3.20). The results indicate that while RU486 function is modulated by the hormonal milieu, it may not be to the same extent as that of Anastrozole. Furthermore, the results indicate that the hormonal milieu results in differential effects on MUC1 nuclear and cytoplasmic localisation as opposed to RU486.

Investigations of MUC1 expression in the combined hormone-therapy and immunotherapy groups revealed that the addition of NK cell immunotherapy to RU486 treatment (RU486+NK) caused a significant decrease ($p < 0.001$) in MUC1 MFI in both the nucleus and the cytoplasm compared with the diluent control (Figure 3.24). While the addition of hormones (EPR+NK) followed this trend, in this group MUC1 was significantly higher than that of the RU486+NK treatment group alone and the hormone control (EP) (Figure 3.24). These results contradicted visual observations in which all cells within the combined hormone-therapy and immunotherapy groups evidenced higher cytoplasmic MUC1 fluorescence (Figure 3.20) and distinct nuclear localisation compared to the control groups (Figure 3.19). Again, it is suggested that quantitative analysis of large data sets is more ideal for interpretation than qualitative

observations. The results furthermore, are reminiscent of the effects of NK cell mediated Anastrozole treatment, where the combined influences of NK cells and hormonal stimuli increase MUC1 expression.

We further compared the effects of hormone-therapy alone (HT) with combined hormone-therapy and immunotherapy (HT+IT) (Figure 3.25). In the RU486 treatment group NK cell mediation resulted in a significant decrease ($p<0.001$) in only cytoplasmic MUC1 MFI. With the addition of a hormonal influence (EPR), NK cell mediation resulted in a significant increase ($p<0.001$) in both cytoplasmic and nuclear MUC1 MFI compared to hormone-therapy alone. This highlights that with hormone-therapy, under hormonal stimuli, NK cells mediate an increase in MUC1 expression in MCF-7 cells.

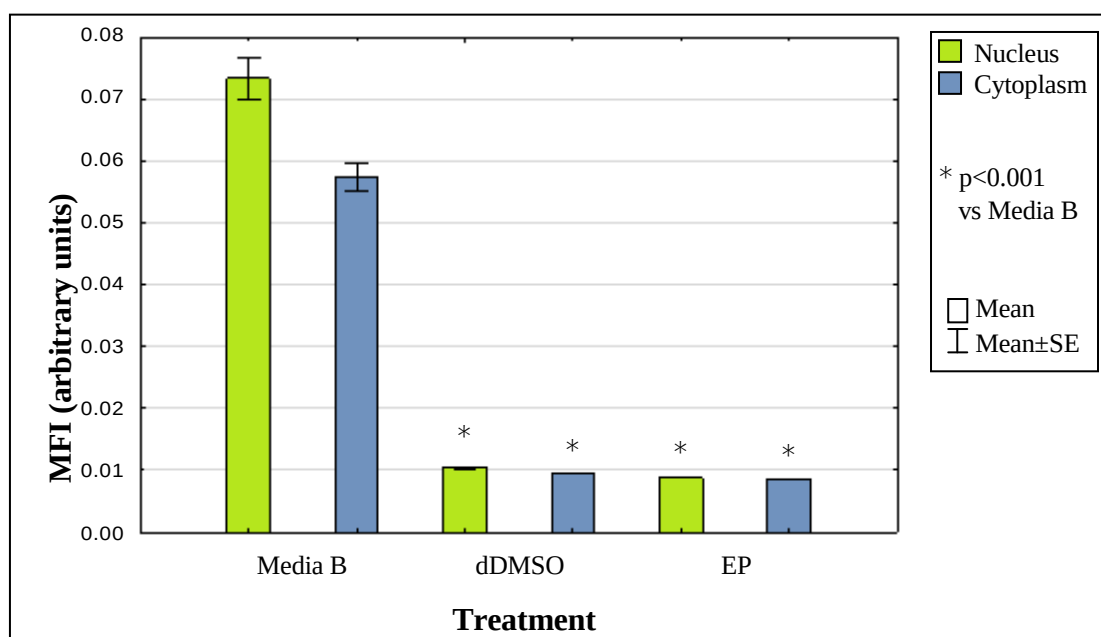


Figure 3.22. A bar graph showing the nuclear and cytoplasmic MUC1 MFI for the control treatments

Both the diluent and hormone control treatments (dDMSO and EP, respectively) induced a significant decrease ($p<0.001$) in MUC1 MFI in both the nucleus and cytoplasm compared to the Media B control. For subsequent analysis since all drugs and hormones were diluted in DMSO, the DMSO control group is used as a baseline comparator group instead of the Media B control.

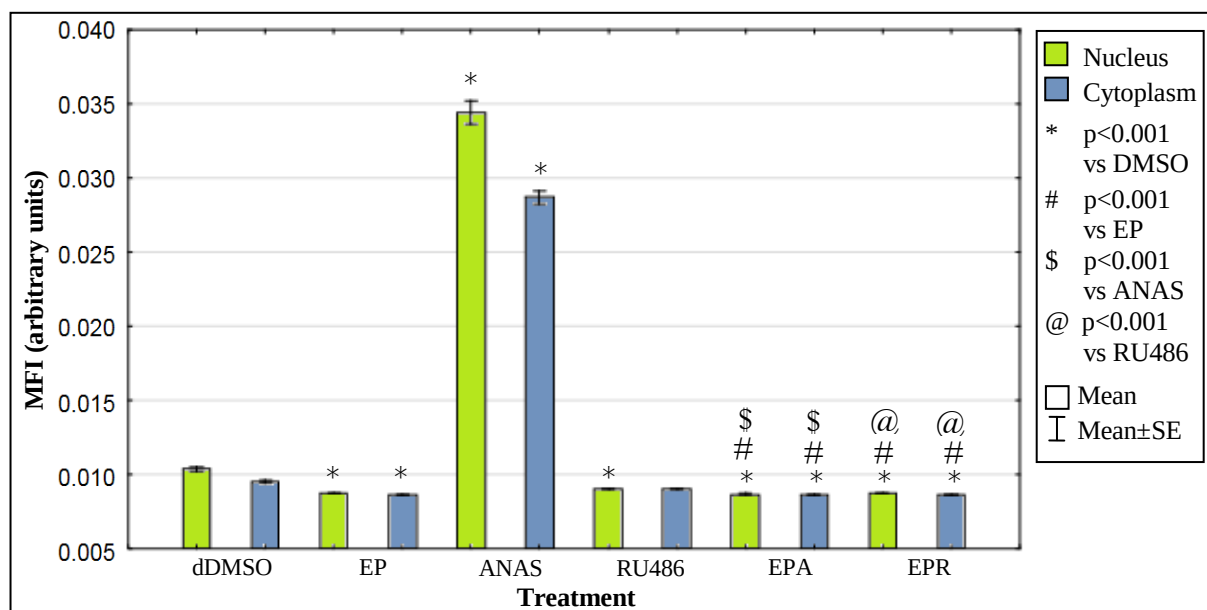


Figure 3.23. A bar graph showing nuclear and cytoplasmic MUC1 MFI for the control and hormone-therapy treatments

Anastrozole treatment (ANAS) induced the highest MUC1 MFI in both the nucleus and cytoplasm as compared to the other HT groups. MUC1 expression was significantly higher ($p < 0.001$) in the ANAS group compared to the diluent control (dDMSO). MUC1 expression in RU486-treated cells was comparatively reduced in the nucleus only ($p < 0.001$) compared to the diluent control. The addition of hormones (EPA) saw a significant reduction in MUC1 MFI compared to the diluent control in both cellular compartments. Compared to the hormone control (EP), both EPA and EPR treatments reduced cytoplasmic MUC1, yet increased nuclear MUC1 ($p < 0.001$). Further, when EPA and EPR were compared to matched samples without hormone influence (ANAS, RU486), it was found that EPA induced a significant reduction in MUC1 cytoplasmic and nuclear expression; while EPR induced a reduction in cytoplasmic MUC1 but a concomitant increase in nuclear expression. This indicates that the hormonal milieu plays an important role in the regulation of both Anastrozole and RU486 function in relation to MUC1 expression.

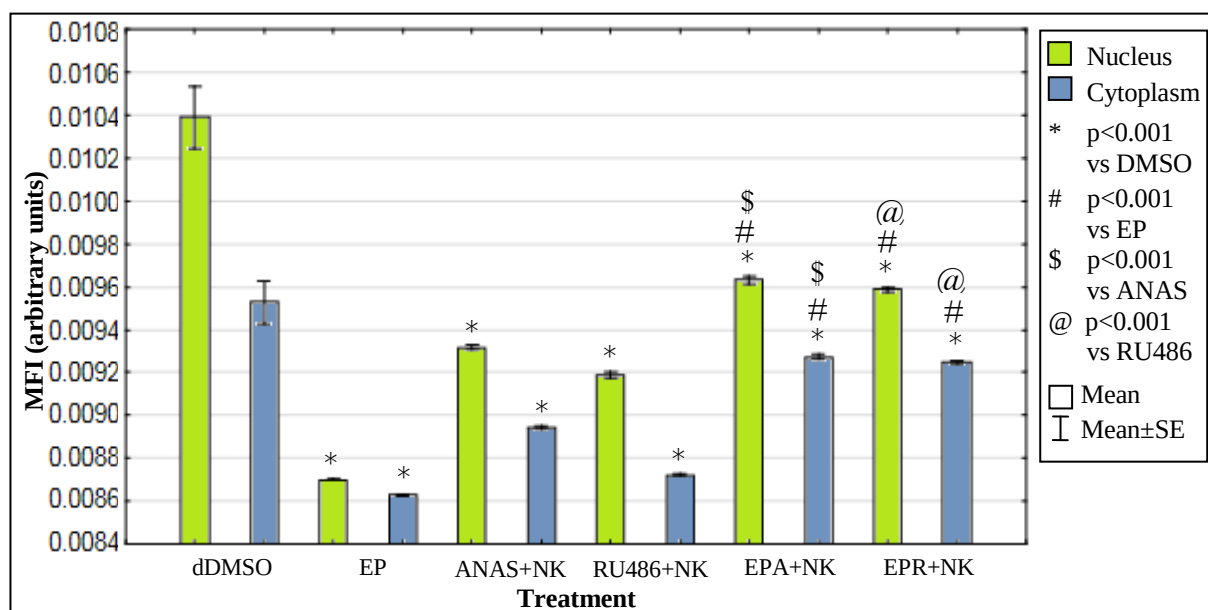


Figure 3.24. A bar graph showing the nuclear and cytoplasmic MUC1 MFI for the control and combined hormone-therapy with immunotherapy treatments

NK cell immunotherapy with both RU486 and Anastrozole treatment alone (RU486+NK, ANAS+NK) and hormone addition (EPR+NK, EPA+NK) induced a significant decrease ($p < 0.001$) in nuclear and cytoplasmic MUC1 MFI compared to the diluent control (dDMSO), and a significant increase ($p < 0.001$) in both cellular compartments compared to the hormone control (EP). Compared to cells treated with NK cells and hormone-therapy alone (ANAS+NK, RU486+NK), the addition of hormones (EPA+NK, EPR +NK)) resulted in significantly higher MUC1 MFI in both cellular compartments. This indicates that in the presence of NK cells, hormone-therapy is affected by hormonal influences in the mediation of MUC1 expression.

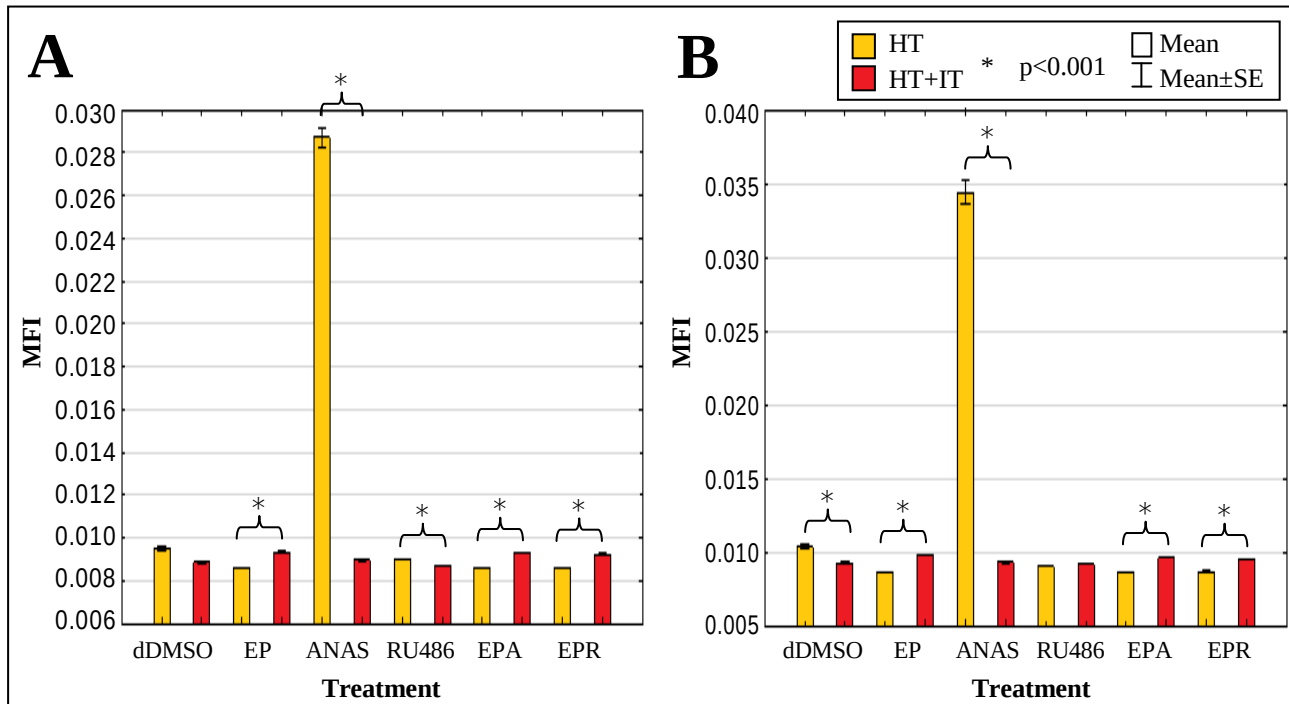


Figure 3.25. Bar graphs showing the cytoplasmic and nuclear MUC1 MFI for the hormone-therapy compared to combined hormone-therapy with immunotherapy treatments

A: Cytoplasm. **B:** Nucleus. Immunotherapy with NK cells (HT+IT, red bars) resulted in a significant ($p < 0.001$) decrease only in nuclear MUC1 MFI in the diluent control group (dDMSO) compared with hormone-therapy alone (HT). Conversely, HT+IT treatment resulted in a significant increase in nuclear and cytoplasmic MUC1 in the hormone control group (EP). In the HT group, Anastrozole induced a notable large increase in MUC1 which was significantly attenuated ($p < 0.001$) by the addition of NK cell therapy (HT+IT). This was mimicked under RU486 treatment, albeit with a reduced level of MUC1 MFI. With the addition of a hormonal milieu, NK cell presence induced an increase ($p < 0.001$) in MUC1 MFI even with Anastrozole treatment (EPA) and RU486 treatment (EPR). The results indicate that MUC1 expression would be affected not only by hormone-therapy, but by the microenvironment (NK cell presence and hormonal stimuli).

Summary of Biomarker Analysis – significant results

The Effects of Control Treatments

Three separate control treatments were identified for use in this study. The Media B control was initially selected as a baseline control for as a cell synchronisation medium. However, it was noted that the other two control groups induced biomarker expression significantly different to that of the Media B control. Specifically, the diluent control, DMSO, in which all hormones and hormone-therapy drugs were diluted, presented significantly higher PR expression, and lower ER α and MUC1 expression in both nuclear and cytoplasmic compartments compared to the Media B control (Figure 3.26). The expression of all biomarkers in EP (hormone control) was significantly lower in both compartments compared to the Media B control group (Figure 3.26). Given the importance of the diluent control in assessing effects due to the hormones and drugs, this control group was selected as a baseline comparator group. Furthermore, when we considered that the EP control (itself diluted in DMSO) simulates an *in vivo* hormonal environment and thus may give additional insight, this control group was retained to investigate the effects of additional hormones on hormone-therapy drugs and NK cell mediation.

The Effects of Anastrozole

Anastrozole treatment decreased PR expression, and increased MUC1 expression in both the nuclear and cytoplasmic compartments. Variable effects were noted on ER α expression based on cellular compartment, being decreased nuclear expression and conversely increased cytoplasmic expression (Figure 3.26). Under hormone mediation, expression of all three biomarkers decreased compared to matched samples without additional hormonal stimuli (Figure 3.26), indicating a particular role for oestrogen and progesterone in ER α , PR and MUC1 expression regulation.

Anastrozole treatment together with NK cell mediation also decreased PR expression and MUC1 expression in both cellular compartments. Differential effects were again noted on ER α expression where nuclear ER α expression decreased and cytoplasmic expression increased (Figure 3.26). The effects seen were similar to that of Anastrozole treatment alone. Additional hormone stimulation, combined Anastrozole treatment with NK cell mediation did not

uniformly affect biomarker expression. Specifically, inducing an increase in nuclear PR and a concomitant decrease in cytoplasmic PR expression; whereas ER α was increased and MUC1 expression conversely decreased in both cytoplasmic compartments (Figure 3.26). This indicates that Anastrozole treatment differentially affects biomarker expression in cellular compartments under NK cell mediation and hormonal stimuli, which better reflects the *in vivo* microenvironment.

A comparison of treatment modalities revealed that the combined therapeutic modality (hormone-therapy and immunotherapy) induced a significant overall decrease in PR expression compared to hormone-therapy alone, regardless of additional hormonal influence (Figure 3.26). This differed to ER α and MUC1, whereby expression was reduced with the combined modality of Anastrozole and NK cell mediation; however, the addition of hormonal stimuli ameliorated the effects of the NK cells, resulting in higher ER α in both cellular compartments (Figure 3.26).

The Effects of RU486

RU486 treatment induced a decrease in PR expression in both nuclear and cytoplasmic compartments. Variable effects were seen on the other biomarkers, with MUC1 nuclear expression decreasing and ER α expression increasing in the cytoplasm (Figure 3.26). With the addition of hormones differential effects were noted on biomarker expression, and cellular localisation. Specifically, there was an increase in PR expression and a converse decrease in ER α in both cellular compartments; whereas MUC1 expression decreased in the cytoplasm and increased in the nucleus (Figure 3.26). This indicates that RU486 treatment not only induces differential effects on biomarker expression in relation to localisation, but that oestrogen and progesterone may indeed alter the effect of this drug.

The addition of NK cells to RU486 hormone-therapy produced a decreased PR and MUC1 expression; however, nuclear ER α expression decreased with a contrasting increase in cytoplasmic ER α expression (Figure 3.26). Under hormonal mediation, RU486 and NK cell mediation resulted in an increase in overall ER α and MUC1 expression, with variable results regarding PR expression which increased in the nucleus and decreased in the cytoplasm (Figure 3.26). This result indicates a definitive role for NK cell mediation of MCF-7 biomarker expression during treatment with RU486.

When comparing the effects of treatment modalities in altering biomarker expression, it was found that the combined therapeutic modality (RU486 hormone-therapy and immunotherapy) resulted in a significant decrease in PR and ER α expression in both cellular compartments, with MUC1 expression decreasing in the cytoplasm only (Figure 3.26). The addition of hormones, together with RU486 and NK cell mediation increased ER α , MUC1 expression in both cellular compartments, and conversely decreased overall PR expression (Figure 3.26). These results again indicate that the effects of the hormonal milieu in relation to immune cell presence may result in variable effects on biomarker expression during RU486 therapy.

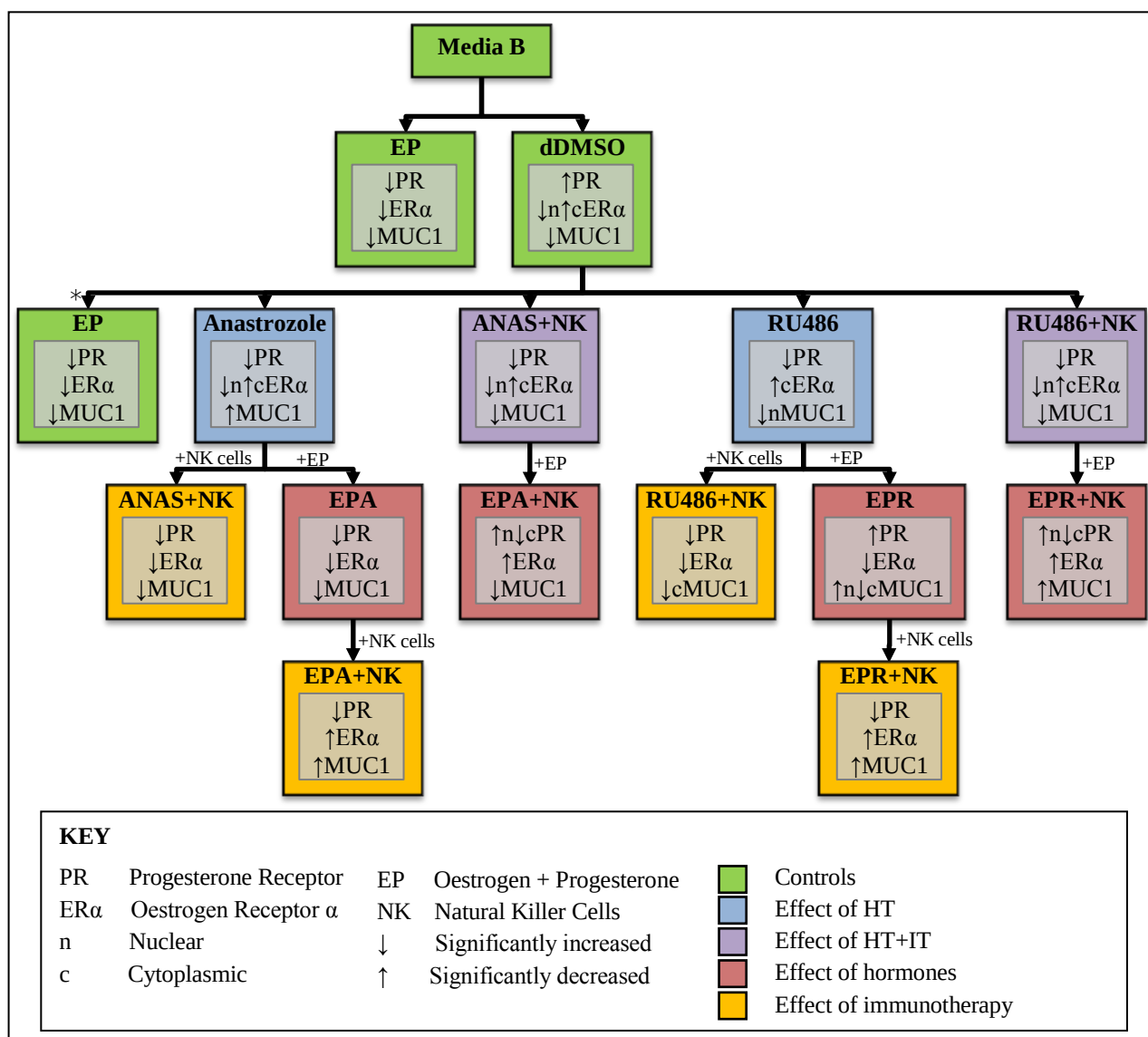


Figure 3.26. A flow diagram summarising the quantitative results for the expression of the three biomarkers

Green boxes: the effects of the controls. Hormone (EP) and diluent (sDMSO) controls compared to the media B control, and hormone (EP) control compared to the diluent (dDMSO) control (*). **Blue boxes:** the effects of hormone-therapy (ANAS and RU486) compared to the dDMSO control. **Purple boxes:** the effects of combined hormone-therapy and immunotherapy (ANAS+NK and RU486+NK) compared to the dDMSO control. **Red boxes:** the effects of hormone therapy and combined therapy, with hormones (EPA, EPA+NK, EPR and EPR+NK) compared to matched group without hormone stimulation (ANAS, ANAS+NK, RU486, RU486+NK). **Orange boxes:** the effects of combined hormone-therapy and immunotherapy (ANAS+NK, RU486+NK, EPA+NK and EPR+NK) compared to hormone-therapy alone (ANAS, RU486, EPA, EPR)

The control treatments show a trend of a general decrease in all three biomarkers expression (green boxes). The hormone-therapy (HT) and combined therapy (HT+IT) treatments show a general decrease in PR and MUC1 expression with an increased cytoplasmic ERα and decreased nuclear ERα expression (blue and purple boxes). The addition of hormones (+EP) to hormone-therapy decreased ERα and MUC1 with variable effects on PR dependent on the hormone-therapy used (red boxes). The addition of hormones (+EP) to the combined therapy increased ERα and nuclear PR and decreased cytoplasmic PR, with variable effects on MUC1 dependent on the hormone-therapy used (red boxes). The addition of immunotherapy (+NK) decreased PR expression; however, the effect on ERα and MUC1 was dependent on the presence of hormones (orange boxes).

Co-localisation of MUC1 with hormone receptors

The assessment of co-localisation of the MUC1 and ER α proteins, and the MUC1 and PR proteins can only be done described qualitatively from the ICC images, which as previously mentioned is a subjective analytical tool. CellProfiler does not lend itself to such image analysis, since it requires all fluorescence data to be converted to greyscale. As such, attempting to ascertain co-localisation and simultaneously distinguishing between two fluorescent markers would not be possible. Thus for qualitative assessment of co-localisation of biomarkers, to reiterate, PR and ER α were localised with antibodies conjugated to Alexa Fluor 488, producing a green fluorescence, while MUC1 was localised with an antibody conjugated to Alexa Fluor 594, producing a red fluorescence. These images were also then overlaid with DAPI, a nuclear stain, producing a blue fluorescence. As such co-localisation of MUC1 and PR, or ER α overlaid with DAPI images produces an interesting array of colours. MUC1 nuclear dominance is visualised as reddish/purple staining due to overlap of MUC1 (red fluorescence) with DAPI (blue fluorescence). ER α or PR nuclear dominance is visualised as a pale blueish/green due to overlap of green fluorescence with DAPI. In the cytoplasm, overlap of MUC1 (red) and ER α or PR (green) is indicated by variation from red/yellow fluorescence (MUC1 dominance) to yellow/green (ER α or PR dominance).

In order to investigate further whether observed co-localisation is indicative of a relationship between the biomarkers, the data obtained was analysed using the Spearman's rank order correlation test. Specifically, this test was conducted to determine if there was a correlation between the MUC1 and each hormone receptor that varied with treatment. The correlation coefficient, Spearman's rho (r_s), generated would give an indication of how the biomarkers co-vary. Specifically an $r_s=1$ is indicative of a perfect positive relationship, with biomarkers co-varying in expression together; an $r_s=0$ indicates no relationship, while an $r_s=-1$ is indicative of an inverse relationship. As such, r_s scores in between 0 and 1 (or -1 and 0), indicate weak correlation ($r_s < 0.5$), low correlation ($r_s < 0.6$) and strong correlation ($r_s > 0.8$) (Adapted from Mukaka, 2012). For the correlation graphs see Appendix C Figures C2-C7, and the table of correlation values are in Appendix D Table D19.

MUC1 and PR co-localisation

The cells treated with the controls (media B, DMSO diluent and EP) show a dominant MUC1 expression in the nucleus (Figure 3.27A), corroborated by individual staining of MUC1 (Figure 3.19). MUC1 nuclear dominance is visualised by the extensive reddish/purple nuclei which stain as such due to the overlap of red fluorescence (MUC1) with DAPI (blue). MUC1 also dominated the cytoplasm (red fluorescence), with PR localisation appearing as green spots, or lighter yellow/green areas. In the Media B control, in which cells underwent cell cycle synchronisation, MUC1 dominated the cytoplasm (red fluorescence), with only some cell showing higher PR intensity (green) (Figure 3.27A). Co-localisation of MUC1 and PR in the hormone control group (EP) showed low, yet dominant nuclear PR expression evinced by pale green/blue nuclei (Figure 3.27B). MUC1 cytoplasmic dominance was more evident in this group, with few cells showing yellow/green fluorescence indicating a shift to PR expression (Figure 3.27B). Conversely in the diluent control group, the majority of nuclei are MUC1 positive, with the cytoplasmic compartment showing MUC1 expression primarily in the perinuclear region, with PR dominating the cytoplasm as shown by the excess of yellow/green staining (Figure 3.27C). Individual images of PR and MUC1 alone, affirm these observations (Figure 3.5, Figure 3.19).

Spearman's rank order test reveals poor correlations between MUC1 and PR expression in the Media B control group in both the nucleus ($r_s=0.27$) and cytoplasm ($r_s=0.25$) (Appendix D, Table D19). While the cells in the dDMSO treatment group conversely showed a moderate correlation in the nucleus ($r_s=0.64$) and cytoplasm ($r_s=0.64$); only EP treatment produced a higher moderate nuclear correlation ($r_s=0.74$) and a moderate cytoplasmic correlation ($r_s=0.54$) (Appendix D, Table D19). Overall these results indicate that under the control treatments, no definitive correlation between these biomarkers expression exists.

All of the hormone-therapy treatments show a greater concentration of MUC1 in the nucleus as demonstrated by reddish/purple nuclei with distinct red spots indicating MUC1 (red) overlap with DAPI (blue) (Figure 3.28). Cells treated with Anastrozole alone, while showing distinct red MUC1 cytoplasmic staining, did in fact show high intensity perinuclear PR (Figure 3.28A). When compared to the diluent control (Figure 3.27C), cytoplasmic PR expression was greatly overwhelmed by MUC1. RU486 treatment induced a definitive mixed cytoplasmic MUC1/PR overlap (red and yellow). In many cells in which PR expression in the cytoplasm was dominant (yellow/green), this was also reflected in the nucleus (pale blue/green) (Figure

3.28B). While these results are similar to that of the diluent control, PR cytoplasmic dominance is notably increased by RU486 treatment compared to the Media B control (Figure 3.27A).

With the addition of hormones, cells treated simultaneously with Anastrozole (EPA) showed a definitive increase in mixed cytoplasmic MUC1/PR expression (red and yellow) (Figure 3.28C). Compared to treatment with Anastrozole alone, cell cytoplasm showed greater PR expression primarily in the perinuclear region (Figure 3.28A, C). Compared to the hormone control (EP) (Figure 3.27B), PR nuclear localisation was distinctly reduced in the EPA group (Figure 3.28C). In the EPR group, in which cells were treated with hormones and RU486, mixed cytoplasmic MUC1/PR expression (red, yellow) was noted, with no distinct dominance of either biomarker (Figure 3.28D). Compared to the hormone control (Figure 3.27B), PR expression in the nucleus was greatly reduced by EPR treatment (Figure 3.28D). This may indicate that treatment with Anastrozole or RU486 in conjunction with hormonal stimuli or not, effectively induces the translocation of PR to the cytoplasm.

The qualitative observations are better explained by the results of the Spearman's rank order correlation test, which show that Anastrozole and RU486 treatment induces a strong correlation ($r_s=0.84$, $r_s=0.92$ respectively) between cytoplasmic MUC1 and PR. While a strong correlation between these biomarkers is maintained under RU486 treatment in the nucleus ($r_s=0.87$), under Anastrozole treatment this is reduced to only a moderate association ($r_s=0.69$) (Appendix D, Table D19). With the addition of hormones, a strong correlation between MUC1 and PR is upheld with combined Anastrozole treatment (EPA) in the cytoplasm only ($r_s=0.795$). Biomarker correlation in the nucleus of cells in this treatment group becomes much weaker ($r_s=0.56$). Interestingly, simultaneous treatment with RU486 and hormones maintains a strong correlation between MUC1 and PR in the nucleus ($r_s=0.87$), with a drastic loss of correlation in the cytoplasm ($r_s=-0.2$) (Appendix D, Table D19). These results show firstly, that correlation between MUC1 and PR is dependent on cellular compartment; secondly, that hormone-therapy can modulate the association between the markers; and lastly, that this modulation is itself dependent on the hormonal milieu.

With the addition of immunotherapy (HT+IT) treated cells all exhibit dominant PR expression in the nucleus as shown by the pale blue nuclei (Figure 3.29). This is correlated with the individual images of PR, apart from the RU486+NK image (Figure 3.7B) which conversely shows very little expression of PR. Overall, the addition of immunotherapy resulted in an essentially 'even' contribution between MUC1 and PR, although a shift to the red spectrum i.e.

MUC1 was noted in those groups under immune mediation with Anastrozole and RU486 (Figure 3.29A, B). The even spread of mixed MUC/PR is due to the individual intermediate cytoplasmic PR expression (Figures 3.7A, 3.7C) and intermediate to high MUC1 expression (Figures 3.21A, 3.21C) initially presented. With the continuation of a hormonal milieu, it appears that cells under both Anastrozole and RU486 treatment (EPA, EPR) (Figure 3.28C, D), expressed a slight dominance of PR as visualised by a more yellow/green fluorescence. These alterations indicate more strongly, that a dramatic alteration in PR expression occurs under immune mediation compared to matched samples without immune mediation (Figure 3.28).

Spearman's correlation produces astonishing results. Under immune-mediation, in the nuclear compartment, MUC1 and PR have a strong correlation in all treatment (HT+IT) groups ($r_s > 0.8$), particularly under RU486 treatment (Appendix D, Table D19). Greater variation is seen in the cytoplasmic compartment, where under NK cell influence Anastrozole (ANAS+NK) induces only a moderate correlation ($r_s = 0.64$) between the two biomarkers, while RU486 (RU486+NK) induces a very strong correlation ($r_s = 0.91$). With the addition of hormonal stimuli, MUC1 and PR show a strong correlation ($r_s = 0.84$) under Anastrozole treatment (EPA), and conversely, under RU486 treatment become only moderately associated ($r_s = 0.77$) (Appendix D, Table D19). These results corroborate suppositions implied by qualitative observations that MUC1 and PR expression may co-vary and alter not only under treatment but with mediation from hormonal stimuli and immune cells.

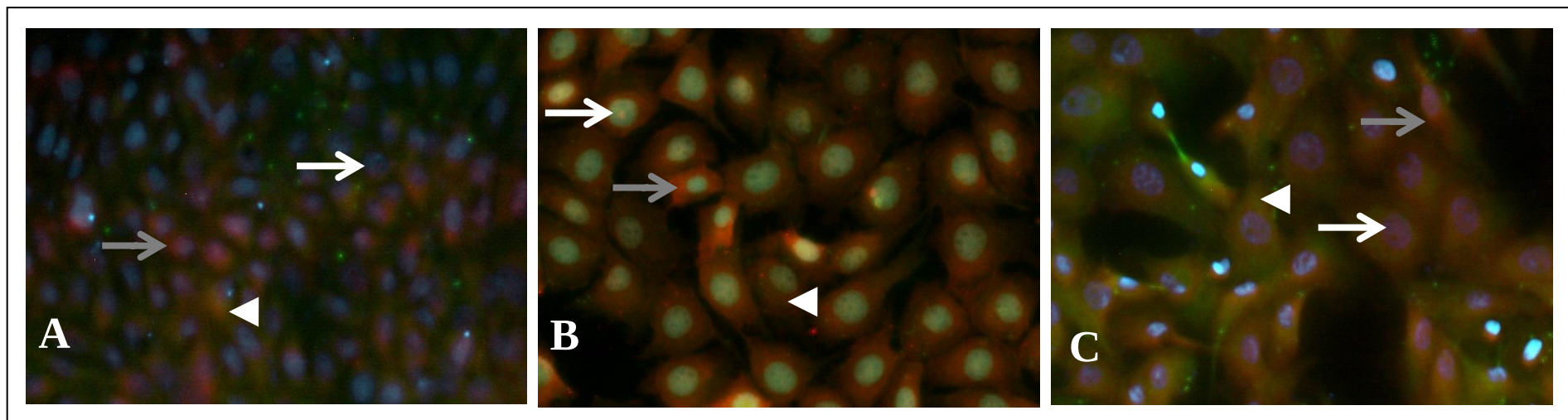


Figure 3.27. Representative photomicrographs showing the effects of control treatments on co-localisation of MUC1 and PR in MCF-7 cells

Control treatment groups: **A:** Media B. **B:** EP. **C:** dDMSO. Fluorescent microscopy, 40x magnification. MUC1 nuclear dominance is visualised as reddish/purple staining due to overlap of MUC1 (red fluorescence) with DAPI (blue fluorescence). PR nuclear dominance is visualised as a pale blueish/green due to overlap of PR (green fluorescence) with DAPI. In the cytoplasm, overlap of MUC1 (red) and PR (green) is indicated by variation from red/yellow fluorescence (MUC1 dominance) to yellow/green (PR dominance) **A:** MCF-7 cells treated with Media B show a high intensity of nuclear (white arrow, reddish/purple) as well as cytoplasmic MUC1 (grey arrow, red), with a few cells showing a higher cytoplasmic PR expression (arrowhead, yellow/green). **B:** Cells treated with the hormone control (EP) show pale blue/green nuclei (white arrow), some of which demonstrate distinct red spots indicating MUC1 localisation. Overall a dominant cytoplasmic MUC1 expression (grey arrow, red) is noted, with few areas showing a yellow/green fluorescence indicative of low PR expression (arrowhead). **C:** The dDMSO treatment induced high nuclear MUC1 expression as evidenced by reddish/purple nuclei with red spots (white arrow). In the cytoplasm, while many cells showed distinct MUC expression (grey arrow, red), PR expression appeared to dominate this region (arrowhead, yellow/green).

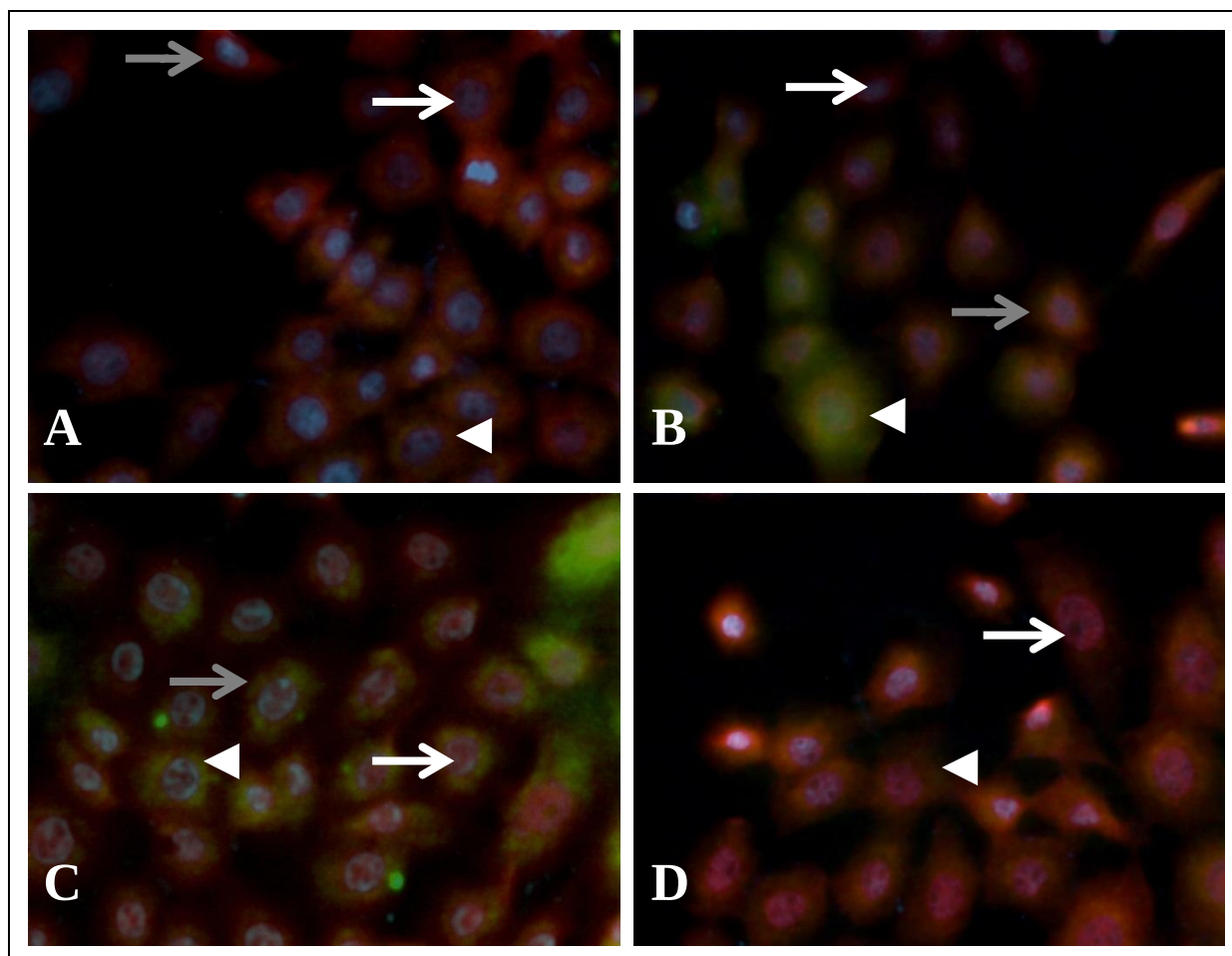


Figure 3.28. Representative photomicrographs showing the effects of hormone-therapy on co-localisation of MUC1 and PR in MCF-7 cells

Treatment groups: **A:** ANAS. **B:** RU486. **C:** EPA. **D:** EPR. Fluorescent microscopy, 40x magnification. MUC1 nuclear dominance is a reddish/purple stain due to MUC1 (red fluorescence) overlap with DAPI (blue fluorescence). PR nuclear dominance, visualised as a pale blueish/green, is due to PR overlap (green fluorescence) with DAPI. In the cytoplasm, overlap of MUC1 (red) and PR (green) is indicated by variation from red/yellow fluorescence (MUC1 dominance) to yellow/green (PR dominance) **A:** Anastrozole treatment induced high nuclear MUC1 intensity (white arrow, reddish/purple nuclei with red spots). MUC1 dominated the cytoplasm (grey arrow, red), with an increase in perinuclear PR (arrowhead, yellow/green). **B:** RU486 treatment also induced high nuclear MUC1 (white arrow) with mixed cytoplasmic MUC1/PR expression (grey arrow, red and yellow). Many cells showed dominant cytoplasmic PR (arrowhead, yellow/green) and simultaneous nuclear PR (pale blueish/green). **C:** In the EPA group, the majority of cells showed high MUC1 nuclear intensity (white arrow, reddish/purple nuclei with red spots). Similar to the Anastrozole only treatment, cells showed PR perinuclear dominance, albeit more extreme. Overall mixed cytoplasmic PR/MUC1 staining (grey arrow, red and yellow/green) with noted PR dominance was seen. **D:** Cells in the EPR group demonstrated dominant nuclear MUC1 (white arrow, reddish/purple nuclei). While cytoplasmic expression showed a mixture of MUC/PR (arrowhead, red and yellow), overall MUC1 dominated the cytoplasm.

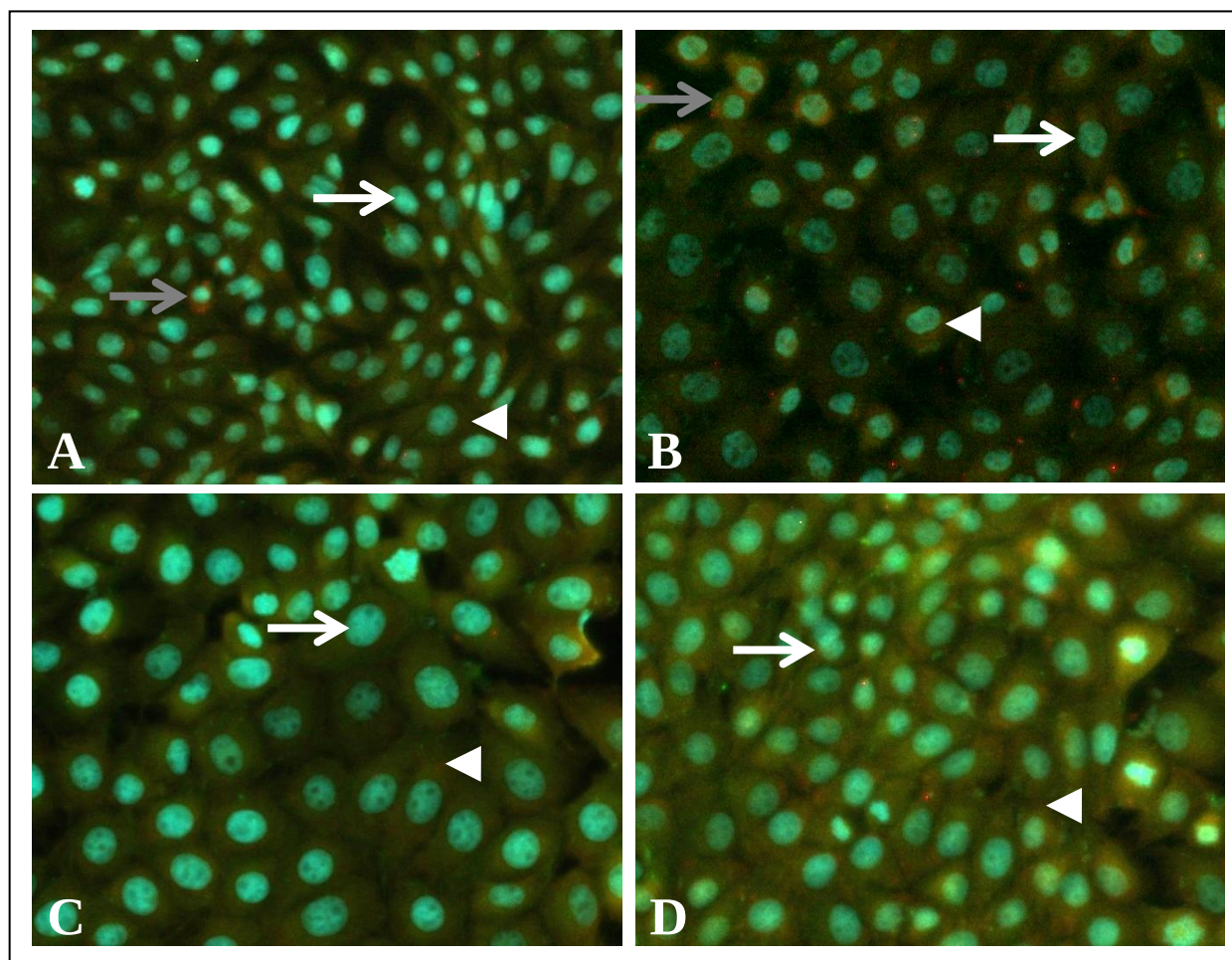


Figure 3.29. Representative photomicrographs showing the effects of combined hormone-therapy and immunotherapy on co-localisation of MUC1 and PR in MCF-7 cells

Treatment groups: **A:** ANAS+NK. **B:** RU486+NK. **C:** EPA+NK. **D:** EPR+NK. Fluorescent microscopy, 20x magnification. PR nuclear dominance is visualised as a pale blueish/green due to overlap of PR (green fluorescence) with DAPI (blue fluorescence). In the cytoplasm, overlap of MUC1 (red) and PR (green) is indicated by variation from red/yellow fluorescence (MUC1 dominance) to yellow/green (PR dominance). In all treatment groups, NK cell mediation resulted in cell nuclei presenting definitive PR dominance (white area, pale blue nuclei). **A:** Cells in the ANAS+NK group show mixed cytoplasmic MUC1/PR (arrowhead, red and yellow) **B:** In the RU486+NK group the majority of cells show mixed MUC1/PR cytoplasmic expression (arrowhead, red/yellow), with few cells showing dominant MUC1 (grey arrow, red) few cells exhibit a higher intensity of nuclear PR with higher cytoplasmic MUC1. **C:** The EPA+NK treated cells show a similar mixed MUC1/PR cytoplasmic expression (arrowhead) with some cells showing a shift to PR dominance. **D:** Cells treated with EPR+NK also exhibit mixed MUC1/PR expression but a slight dominance in PR expression is suggested by the increase in yellow/green fluorescence (arrowhead).

MUC1 and ER α Co-localisation

In the control treatment groups, dominant expression of ER α was noted in the nucleus, primarily in the Media B treatment (cell synchronisation media) and EP treatment (hormone control) (Figures 3.30A, B). However, a distinct cytoplasmic difference was noted in the Media B control, with a higher, more even association of MUC1 and ER α visualised as a red/green fluorescence (Figure 3.30A). In the EP control group, the fluorescence was extremely muted, with some cells showing a marginally higher level of MUC1 as opposed to ER α , and vice versa (Figure 3.30B). The diluent control (dDMSO) showed a dominant MUC1 cytoplasmic expression, visualised as red/orange (Figure 3.30C). Notably, enhanced ER α was associated with the perinuclear region primarily. In this treatment group, nuclei were predominantly MUC1 dominant (reddish blue), with few cells showing the pale blue nuclei associated with ER α prominence (Figure 3.30C). Analysis of the correlation between MUC1 and ER α shows that Media B treatment induced a strong association between the two biomarkers in the cytoplasm ($r_s=0.82$) (Appendix D, Table D19). However, in the same treatment the correlation in the nucleus, was moderate ($r_s=0.75$). The EP control induced only moderate correlations in both nuclear ($r_s=0.65$) and cytoplasmic ($r_s=0.72$) compartments. Interestingly, cells in the diluent control showed weak positive correlation between MUC1 and ER α in the nucleus ($r_s=0.33$) and a weak negative correlation in the cytoplasm ($r_s=-0.32$) (Appendix D, Table D19).

In the hormone-therapy groups, cytoplasmic biomarker expression for cells treated with Anastrozole alone (ANAS) appear evenly spread (Figure 3.31A). Areas of MUC1 dominance (red/green) and ER α dominance (green) were noted (Figure 3.31A); however, staining was considerably reduced compared to the diluent control and media B control (Figure 3.30A, C). With additional hormonal stimuli (EPA) a distinct shift to the red spectrum is noted, indicative of prominent MUC1 cytoplasmic staining (Figures 3.31C). Nuclear expression in both ANAS and EPA treatment groups was difficult to assess, with nuclear biomarker expression obscured by the DAPI stain (Figures 3.31A, C). While individual staining shows low ER α nuclear expression (Figures 3.13A); conversely, the MUC1 single staining image (Figure 3.13C) indicates a higher nuclear expression. Furthermore, quantitative assessment of data showed a significantly higher nuclear expression compared to cytoplasm expression for both biomarkers (Figures 3.16, 3.23). Therefore, we propose that the DAPI stain is obscuring relevant nuclear biomarkers expression causing the co-localisation analysis to be ineffective. When assessing

whether MUC1 and ER α expression co-varied, it was found that under Anastrozole treatment no correlation could be made (nuclear $r_s = -0.10$, cytoplasmic $r_s = 0.09$) (Appendix D, Table D19). With additional hormonal interaction, Anastrozole treatment (EPA) appeared to induce a comparatively increased, yet low correlation in both the nuclear ($r_s = 0.51$) and cytoplasmic compartments ($r_s = 0.53$).

In the hormone-therapy group, cells treated with RU486 show a dominant ER α expression in both the nucleus (pale blue staining) and the cytoplasm (green fluorescence) (Figure 3.31B). Individual staining of ER α (Figures 3.13B) and MUC1 (Figure 3.20B) affirms this result, while quantitative assessment shows that this treatment induced the highest ER α nuclear and cytoplasmic expression (Figure 3.16) and low nuclear and cytoplasmic MUC1 expression (Figure 3.23). Interestingly, the addition of simultaneous hormones (EPR) induced a dominant MUC1 nuclear (reddish blue nuclei with red spots) and cytoplasmic expression (red fluorescence) (Figure 3.30D), as noted in single staining images (Figure 3.13D, Figure 3.20D). The quantitative analysis also corroborates the co-localisation result, with the ER α MFI higher than the MUC1 MFI (Appendix D, Table D7, and D13) for both the nucleus and the cytoplasm. Analysis of the relationship between MUC1 and ER α produced interesting results: RU486 treatment alone produced a very strong ($r_s = 0.92$) and strong ($r_s = 0.79$) correlation between the biomarkers in the nucleus and cytoplasm respectively (Appendix D, Table D19). However, the addition of hormones to treatment (EPR) obliterated this association in both nuclear ($r_s = -0.24$) and cytoplasmic compartments ($r_s = -0.10$). These results indicate that Anastrozole and RU486, even with hormonal stimuli, induce alterations in the MUC-ER α relationship.

With the addition of immunotherapy (HT+IT), all treatments induced a dominant expression of ER α in the nucleus of the cells (Figure 3.32), corroborated by single staining images and data analysis thereof (Figure 3.14, Figure 3.17). Under NK cell mediation, both Anastrozole and RU486 treatment induced dominant MUC1 cytoplasmic expression in the majority of the cells, visualised as red/yellow fluorescence, with a few cells expressing dominant ER α expression (green fluorescence) (Figures 3.32A, B). In comparison with the diluent (dDMSO) control, MUC1 localisation was markedly reduced (Figure 3.30).

With the addition of hormonal stimuli to combined hormone-therapy and immunotherapy, a shift to dominant cytoplasmic ER α expression in the cells treated with Anastrozole (EPA+NK), and shift to a dominant MUC1 expression in cells treated with RU486 (EPR+NK) is noted (Figure 3.32C, D). Notably for all treatments in the hormone-therapy and immunotherapy

groups, strong correlation ($r_s > 0.8$) between MUC1 and ER α was found in both the nucleus and cytoplasm (Appendix D, Table D19). Results found in hormone-therapy treatments without NK cell influence, indicate that the actions of Anastrozole and RU486 vary with the addition of hormonal stimuli; however, that a correlation is maintained between the biomarkers under NK cell mediation, indicates that immune cells have a strong influence on the relationship between MUC1 and ER α .

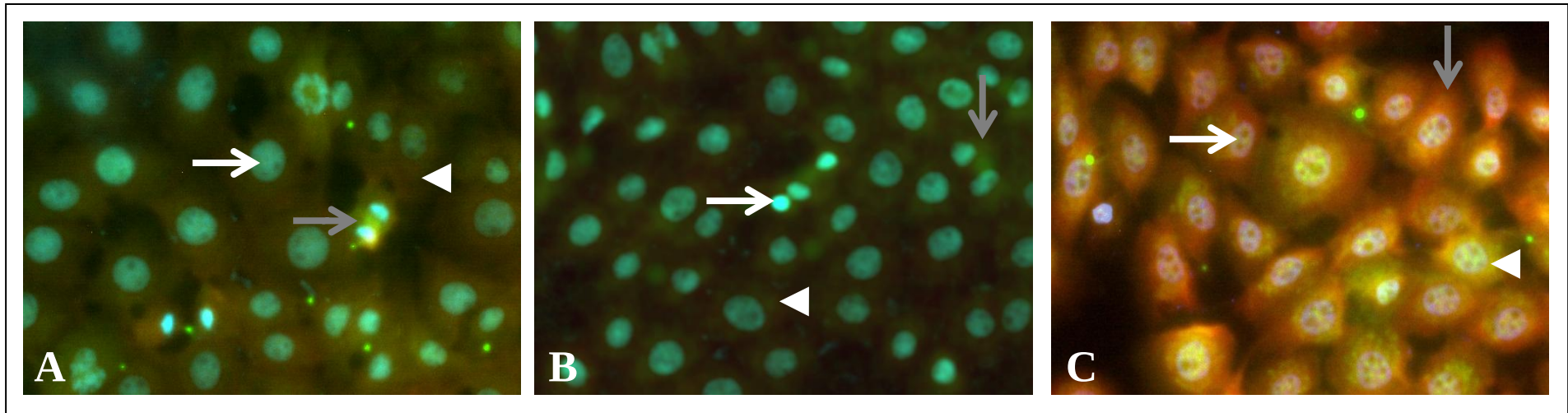


Figure 3.30. Representative photomicrographs showing the effects of control treatments on co-localisation of MUC1 and ERα in MCF-7 cells

Control treatment groups: **A:** Media B. **B:** EP. **C:** dDMSO. Fluorescent microscopy, 40x magnification. MUC1 nuclear dominance is visualised as reddish/purple staining due to overlap of MUC1 (red fluorescence) with DAPI (blue fluorescence). ERα nuclear dominance is visualised as a pale blueish/green due to overlap of ERα (green fluorescence) with DAPI. In the cytoplasm, overlap of MUC1 (red) and ERα (green) is indicated by variation from red/yellow-orange fluorescence (MUC1 dominance) to yellow/green (ERα dominance) **A:** MCF-7 cells treated with Media B showed pronounced nuclear expression of ERα (white arrow, pale blue nuclei). The cytoplasm has an even expression of ERα (green) and MUC1 (red); however, a cell undergoing mitosis shows ERα dominance (grey arrow, green) **B:** MCF-7 cells treated with the hormone control, EP, show ERα nuclear dominance (white arrow, pale blue nuclei). Despite very muted fluorescence, some cells show MUC1 dominating the cytoplasm (arrowhead, green/red), with yet others showing increased ERα (grey arrow, green). **C:** The diluent control induced pronounced nuclear MUC1 (white arrow, reddish purple) although some cells exhibited dominant nuclear ERα (pale blue nuclei). In the cytoplasm, MUC1 appeared to dominate the cells, seen as orange-red (grey arrow), with ERα more prominent in the perinuclear area (arrowhead, yellow/green).

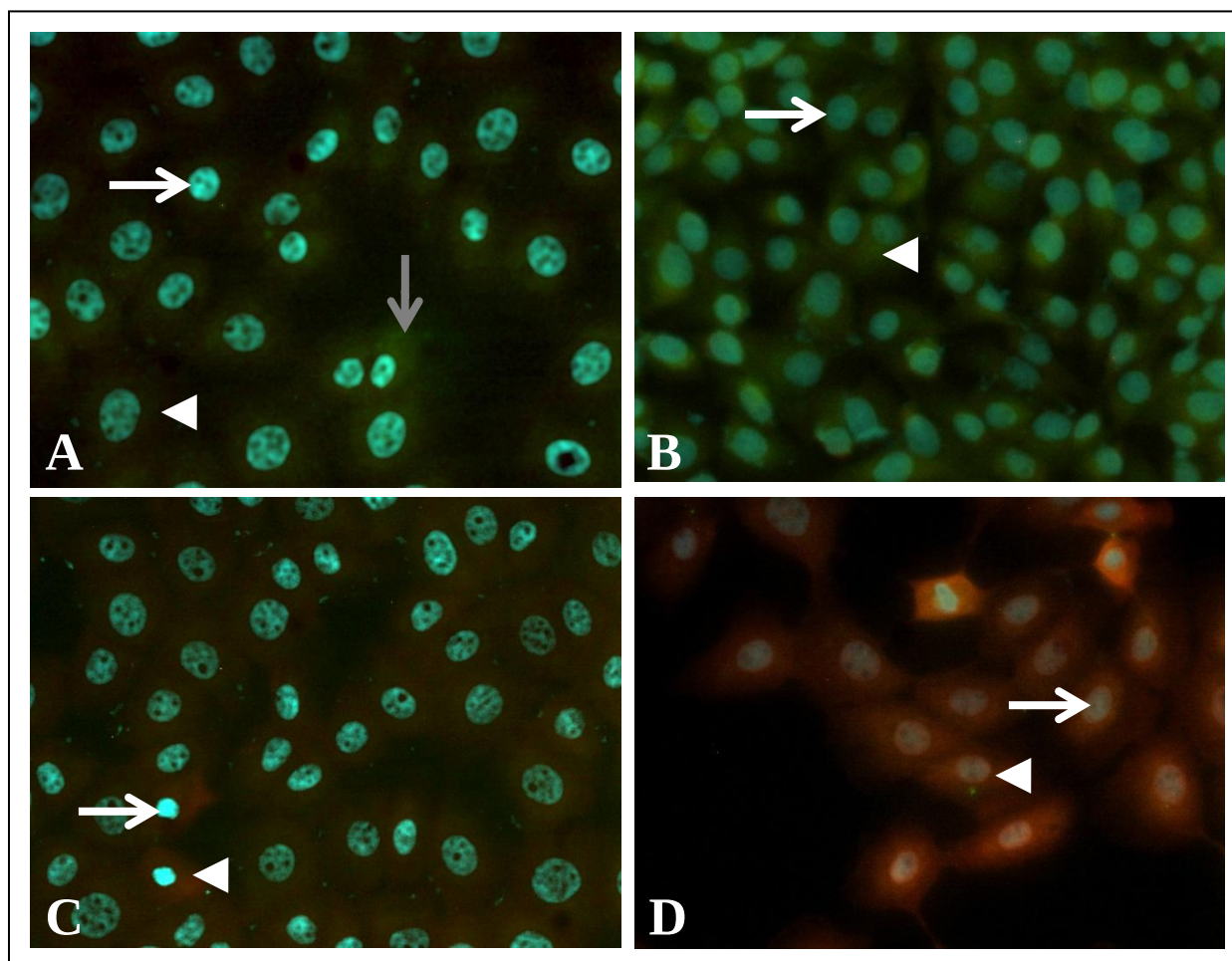


Figure 3.31. Representative photomicrographs showing the effects of hormone-therapy on co-localisation of MUC1 and ER α in MCF-7 cells

Treatment groups: **A:** ANAS. **B:** RU486. **C:** EPA. **D:** EPR. Fluorescent microscopy, 40x magnification. MUC1 nuclear dominance is visualised as reddish/purple staining due to overlap of MUC1 (red fluorescence) with DAPI (blue fluorescence). ER α nuclear dominance is visualised as a pale blueish/green due to overlap of ER α (green fluorescence) with DAPI. In the cytoplasm, overlap of MUC1 (red) and ER α (green) is indicated by variation from red/yellow-orange fluorescence (MUC1 dominance) to yellow/green (ER α dominance) **A:** Anastrozole treatment induced an even expression of both ER α and MUC1 (arrowhead, red/green) in the cytoplasm. Some cells showed a slight dominant ER α fluorescence (grey arrow, green). Nuclear expression was difficult to assess but ER α dominant (white arrow, pale blue) with red spots attributable to MUC1 **B:** MCF-7 cells treated with RU486 show ER α nuclear (arrow, pale blue) and cytoplasmic (arrowhead, yellow/green) dominance. **C:** MCF-7 cells treated with EPA shows a higher cytoplasmic intensity of MUC1 (arrowhead, red) than Anastrozole treatment alone. Nuclear expression is not quite clear but it appears that ER α is dominant. MUC1 localisation is evident as red spots within the nucleus within **D:** EPR treatment induced a very evident MUC1 dominance in both the cytoplasm and the nucleus (arrow, reddish blue). ER α is prominent in the perinuclear region (arrowhead, yellow/red).

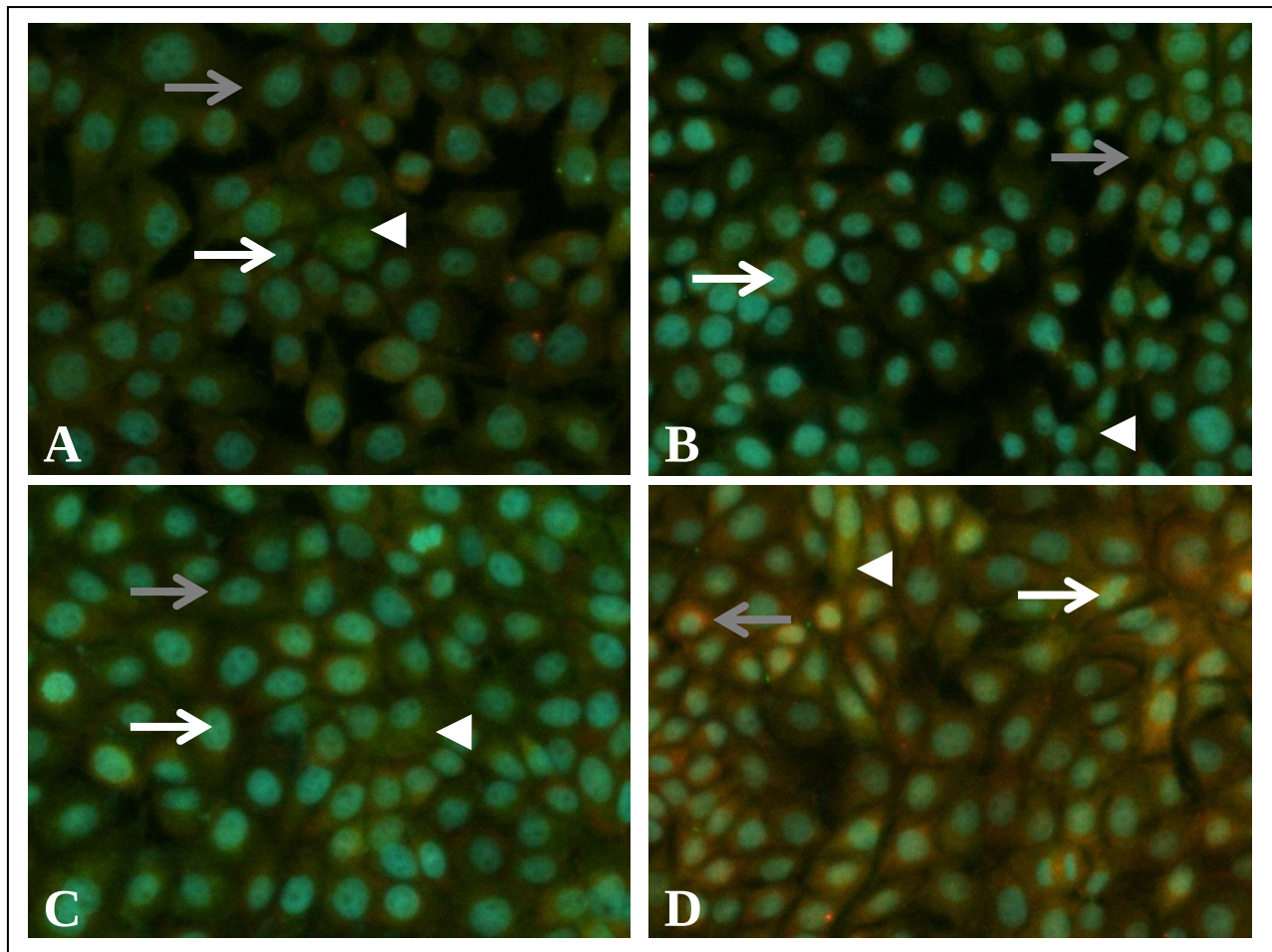


Figure 3.32. Representative photomicrographs showing the effects of combined hormone-therapy and immunotherapy on co-localisation of MUC1 and ER α in MCF-7 cells

Treatment groups: **A:** ANAS+NK. **B:** RU486+NK. **C:** EPA+NK. **D:** EPR+NK. Fluorescent microscopy, 20x magnification. ER α nuclear dominance is visualised as a pale blueish/green due to overlap of ER α (green fluorescence) with DAPI (blue fluorescence). Cytoplasmic overlap of MUC1 (red) and ER α (green) is indicated by variation from red/yellow-orange fluorescence (MUC1 dominance) to yellow/green (ER α dominance). In all treatment groups dominant nuclear ER α (white arrow, pale blue nuclei) was indicated, with some nuclei showing red spots indicative of MUC1 localisation. **A:** The majority of the cells treated with ANAS+NK show a high cytoplasmic MUC1 expression (grey arrow, red/yellow). Some cells exhibited a higher ER α expression (arrowhead, green). **B:** Cells treated with RU486+NK showing a similar spread of cytoplasmic MUC1 dominance (grey arrow, red/yellow) and cytoplasmic ER α dominance (arrowhead, yellow/green). **C:** Compared to the Anastrozole treatment without hormonal influence (ANAS+NK), EPA+NK induced a shift to ER α cytoplasmic dominance was noted (arrowhead, green) with few cells showing higher cytoplasmic MUC1 (grey arrow, red/yellow). **D:** Compared to the RU486 treatment without hormonal influence (RU486+NK), cells treated with EPR+NK show a higher intensity of nuclear ER α (white arrow, pale blue) with a few cells showing typical MUC1 nuclear localisation, evinced by reddish/purple nuclei with red spots. While the majority of cells have dominant MUC1 cytoplasmic expression (grey arrow, red), a few cells have a higher ER α expression (arrowhead, green).

The Effects of Hormone-Therapy and Combined Therapy on Proliferation

Ki67 is a protein found in the nucleus in proliferating cells, it is found during the active phases (G_1 , S, G_2 , and M) of the cell cycle and is absent in the resting phase (G_0) (Scholzen and Gerdes, 2000).

The ICC controls were the following: negative control, primary antibody control for Ki67, secondary antibody control for Alexa Fluor® 488, and isotype control for Ki67. All the ICC controls showed no fluorescence indicating that there was no background fluorescence, no autofluorescence, no non-specific binding of the secondary antibodies, and the specificity of the antibodies (see Appendix C, Figure C1). Therefore, the ICC was effective and the results accurate.

To reiterate, following hormone-therapy and immunotherapy of the MCF-7 cells, nuclei were visualised using DAPI, producing a blue fluorescence, and Ki67 immunolocalised, producing a green fluorescence. Qualitative assessment of the images yielded no meaningful information, thus quantitative analysis of the data acquired by CellProfiler was performed to ascertain whether hormone-therapy or combined hormone-therapy and immunotherapy induced alterations in Ki67 expression. Fluorescence was captured individually as grayscale images. The images were analysed with CellProfiler with a pipeline that firstly located the nuclei and the Ki67 from their respective images. Where Ki67 intersected with a nucleus, that nucleus was labelled Ki67 positive ($Ki67^+$) (Figures 3.33, 3.34). These $Ki67^+$ nuclei were then represented as a percentage of all nuclei to determine the percentage of proliferating cells (Figure 3.23; Appendix D, Table D20).

The normality tests Shapiro-Wilk, Jarque-Bera and Anderson-Darling were performed on the data produced by CellProfiler, with significance set at $p=0.05$. The data was normally distributed ($p>0.05$) (see Appendix D Table D21), therefore, the parametric one-way analysis of variance (ANOVA) was used to compare the hormone therapy (HT) and combined hormone-therapy with immunotherapy (HT+IT) groups. Pairwise comparisons were performed between the groups using Tukey's (default of PAST). The significance for both was set at $p=0.05$ (see Appendix D, Table D22).

No significant differences in Ki67 expression were detected between the control groups (Figure 3.33, Figure 3.35). Within the hormone-therapy group, treatment with Anastrozole or RU486

(ANAS and RU486 groups) (Figure 3.34) induced no significant difference ($p>0.05$) in percentage Ki67⁺ (%Ki67⁺) nuclei compared to the controls (Media B, dDMSO and EP) (Figure 3.35). Furthermore, simultaneous treatment with the hormones, oestrogen and progesterone (EPA and EPR groups), had no effect on the anti-proliferative capacity of the drugs (Figure 3.34, Figure 3.35). These results were mimicked within the HT+IT group, indicating that combined hormone-therapy and immunotherapy had no significant effect on proliferation of MCF-7 cells at 72 hours compared to the controls (Figure 3.35).

We further compared hormone-therapy to combined hormone-therapy and immunotherapy to assess whether the addition of NK cells to the respective treatment groups altered the Ki67 profile. However, no significant difference ($p>0.05$) in %Ki67⁺ nuclei in the HT groups as compared to the HT+IT groups with their respective treatments was found. Consequently, the NK cells had no significant effect when combined with hormone-therapy with regards to inhibiting proliferation of the MCF-7 cells (Appendix D, Table D22). However, NK cell mediation (HT+IT) treatments consistently produced values lower than in the hormone-therapy (HT) treatments indicating a reduced proliferation level.

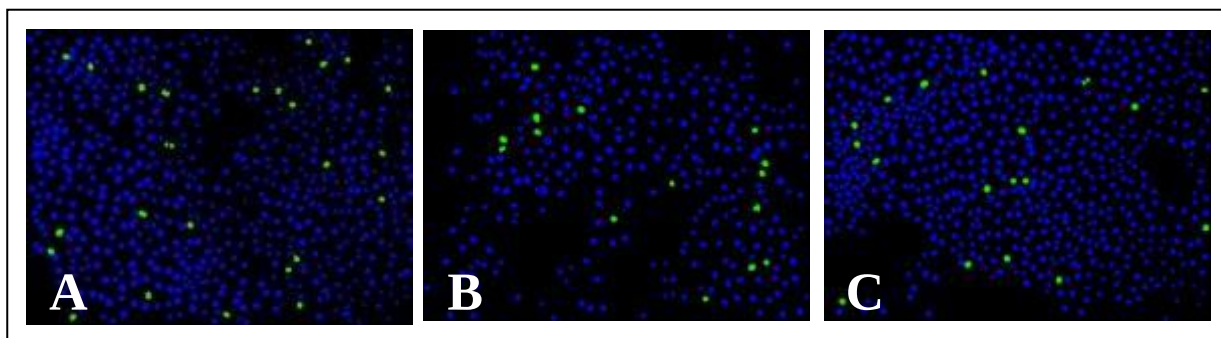


Figure 3.33. Representative CellProfiler composite images showing the effects of control treatments on Ki67 expression

Control groups: **A:** Media **B:** dDMSO. **C:** EP. CellProfiler composite image showing nuclei (blue) and Ki67⁺ nuclei (green). 10X magnification. Control treatments showed no differences in Ki67 expression (Refer to Figure 3.32).

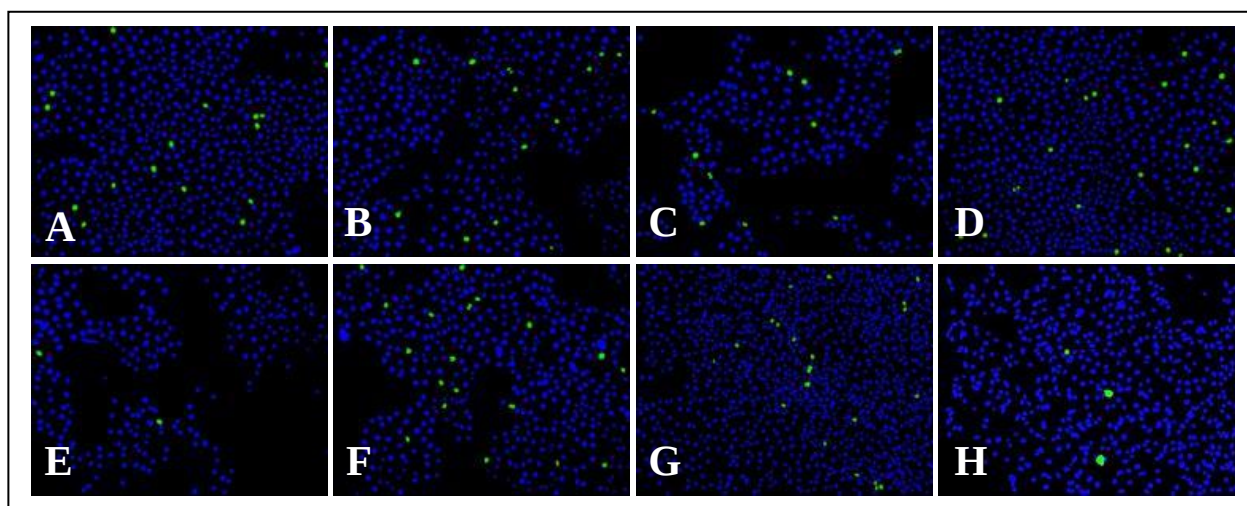


Figure 3.34. Representative CellProfiler composite images showing the effects of hormone-therapy and combined hormone with immunotherapy on Ki67 expression

Treatment groups: **A:** ANAS. **B:** RU486. **C:** EPA. **D:** EPR. **E:** ANAS+NK. **F:** RU486+NK. **G:** EPA+NK. **H:** EPR+NK. CellProfiler composite image showing nuclei (blue) and Ki67⁺ nuclei (green). 10X magnification. While no significant differences in percentage Ki67⁺ nuclei were found (Refer to Figure 3.32), fewer Ki67⁺ nuclei were noted in the ANAS+NK and EPR+NK treatment groups.

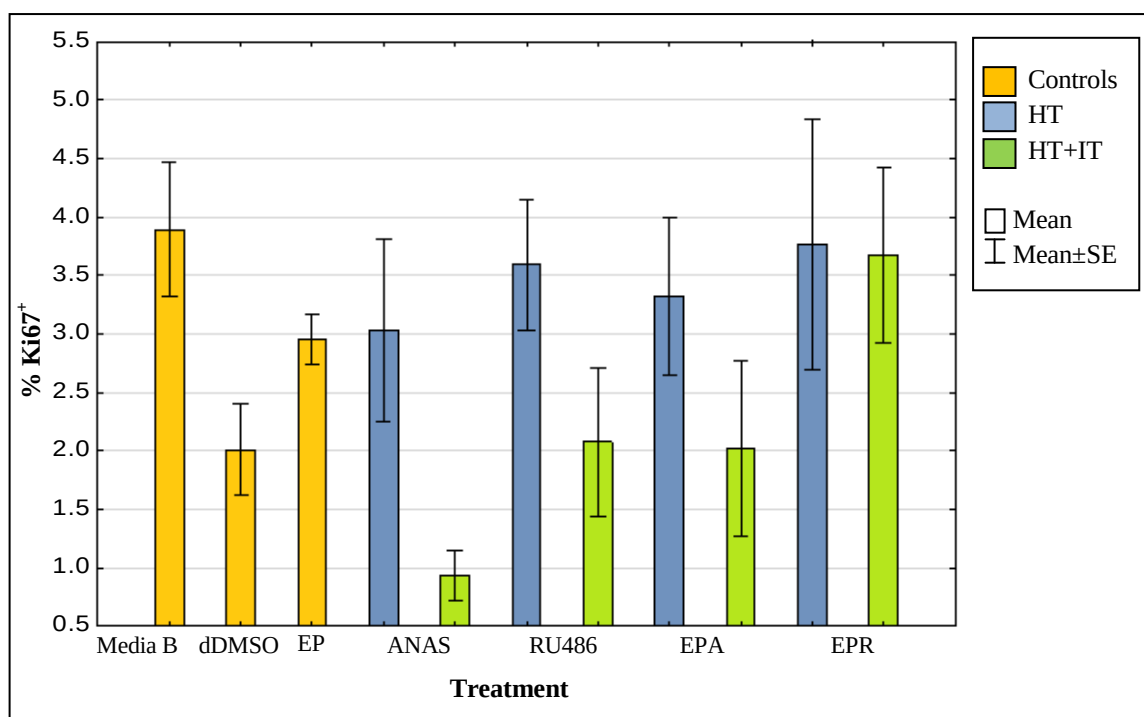


Figure 3.35. A bar graph showing the mean percentage of Ki67⁺ nuclei in the controls, hormone-therapy and combined hormone-therapy with immunotherapy treatment groups

There was no significant difference ($p > 0.05$) in % Ki67⁺ nuclei within and between the control, HT and the HT+IT groups. However, the % Ki67⁺ nuclei in the hormone therapy (HT) groups were consistently higher than in the combined hormone-therapy and immunotherapy (HT+IT) groups. This indicates that NK cells had a positive effect on negating proliferation regardless of treatment.

The Effects of Hormone-Therapy and Combined Therapy on Apoptosis

The flow cytometric Annexin V-FITC apoptosis detection Kit II was used to determine levels of induced apoptosis. During the process of cell death, plasma membrane integrity is lost and the phospholipid, phosphatidylserine, translocates to the extracellular side of the plasma membrane (Cepa *et al.*, 2008). Thus apoptotic events are detected by Annexin V-FITC which binds to phosphatidylserine and propidium iodide (PI) enters the nuclei when the plasma membrane integrity is completely lost.

Data was acquired with BD FACSDiva software v6 and analysed using FlowJo (Free Trial). A graph with side scatter (SSC) on the y-axis (indicating the granularity of the cells) and forward scatter (FSC) on the x axis (indicating the size of the cells), was viewed first (first column of Figure 3.36 - Figure 3.38). Dead cell populations and debris (very small cells with little granularity) were not gated out, as is the usual procedure with other flow cytometric analyses, to determine if there was a difference in the dead cell populations with regards to the different treatments.

Compensation controls were performed to determine and remove any overlap between the FITC and PI emission channels (Appendix C, Figure C8). The camptothecin (CPT) control was used to establish quadrants as follows: quadrant 1 (Q1) Annexin⁺ PI⁻ determining early apoptotic cells, quadrant 2 (Q2) Annexin⁺ PI⁺ showing late apoptotic cells, quadrant 3 (Q3) Annexin⁻ PI⁺ determining necrotic cells, and quadrant 4 (Q4) Annexin⁻PI⁻ showing live cells. The data was then viewed graphically with AnnexinV-FITC on the y-axis and PI on the x-axis.

These quadrants were subsequently applied to all the graphs for the controls (Figure 3.36 second column), hormone-therapy (HT) groups (Figure 3.37 second column) and the combined hormone-therapy with immunotherapy (HT+IT) groups (Figure 3.38 second column).

The percentage of cells in each quadrant was calculated (see Figures 3.36 - 3.38 and Appendix D, Table D23). The normality tests Shapiro-Wilk, Jarque-Bera and Anderson-Darling were performed on the data, with significance set at $p < 0.05$. The majority of the data followed a normal distribution ($p > 0.05$), with the exception of the dDMSO treated Q3 (live cells) and EP treated Q4 (necrosis) data (Appendix D, Table D24). Therefore the non-parametric Kruskal-Wallis one-way analysis of variance was used to compare each treatment with significance set at $p < 0.05$ (Appendix D, Tables D25-D29).

Regarding the control treatment groups, the DMSO diluent control (dDMSO) induced a significant increase ($p < 0.05$) as compared to the Media B control in early apoptosis (Q1) (Figure 3.39) and total apoptosis (Q1+Q2) (Figure 3.43). This indicates that even at low concentrations DMSO has a pro-apoptotic effect. Since DMSO was used as a diluent for all the hormone-therapy drugs and hormones, the dDMSO control was used as the baseline comparator for all further statistical comparisons. The camptothecin (CPT) treatment was used as an apoptotic inducer; however, it did not react as required under the conditions in this study, and instead it reduced early apoptosis comparatively (Figure 3.39). Furthermore, with the hormone control (EP) there was no significant difference in early or late apoptosis as compared to the dDMSO control, but there was a significant decrease ($p < 0.05$) in apoptosis cumulatively (Q1+Q2) (Figure 3.43), indicating that the hormones had a protective effect. Therefore, the EP hormone control was retained as an additional comparator for the treatment groups with continued hormone mediation (EPA, EPR, EPA+NK, EPR+NK).

Importantly, no treatment significantly increased induced apoptosis as would be expected, but rather decreased apoptosis in the vast majority of treatments compared to the diluent control and the hormone control. There was also no significant difference with regards to the percentage of cells undergoing necrosis (Q4), regardless of treatment (HT or HT+IT) (Figure 3.41). However, there was a significant difference with regards to the percentage of live cells (Figure 3.42) with all treatments (apart from EPA).

The Effect of Hormone-Therapy on the Induction of Apoptosis

Anastrozole treatment induced a significant decrease in early apoptosis (Figure 3.39) and total apoptosis (Q1+Q2) compared to the dDMSO control (Figure 3.43). However, under hormone mediation there was no significant difference noted in any quadrant as compared to the baseline dDMSO control, EP hormone control and Anastrozole alone (Figures 3.39 – Figure 3.43) (Appendix D, Table D25, Table D29).

Mimicking the Anastrozole results, RU486 treatment induced a significant decrease ($p < 0.05$) in early apoptosis (Figure 3.39) and total apoptosis (Q1+Q2) (Figure 3.43). This was further repeated with the treatments under hormone mediation also inducing a significant decrease ($p < 0.05$) in early apoptosis (Figure 3.39) and total apoptosis (Q1+Q2) as compared to the dDMSO control (Figure 3.43) (Appendix D, Table D25, Table D29). However, there was a significant decrease ($p < 0.05$) with RU486 treatment under hormone mediation in early apoptosis as compared to the EP hormone control (Figure 3.39). The hormone mediation (EPR), however, had no significant effect ($p > 0.05$) when compared to hormone-therapy alone (RU486) (Appendix D, Table D25, Table D29).

The Effect of Combined Hormone-Therapy and Immunotherapy on the Induction of Apoptosis

With the addition of immunotherapy (NK cells), there is a similar pattern to that of hormone-therapy alone. Anastrozole and RU486 induced a significant decrease ($p < 0.05$) in early apoptosis (Figure 3.39) and total apoptosis (Q1+Q2) as compared to the dDMSO control (Figure 3.43) (Appendix D, Table D25, Table D29). With continued hormone mediation, RU486 induced a significant decrease in early apoptosis (Figure 3.39) while Anastrozole induced a significant decrease in late apoptosis (Figure 3.40) and both significantly decreased total apoptosis (Figure 3.43) as compared to the dDMSO control. However, no significant difference was noted when compared to the EP hormone control or the drugs (under NK cell mediation) regardless of hormone mediation. Interestingly, no significant difference was noted with the comparison of the combined therapy to the hormone-therapy alone regardless of treatment (Appendix D, Table D25, Table D29). This indicates that the NK cells had no effect on the MCF-7 cells regarding apoptosis.

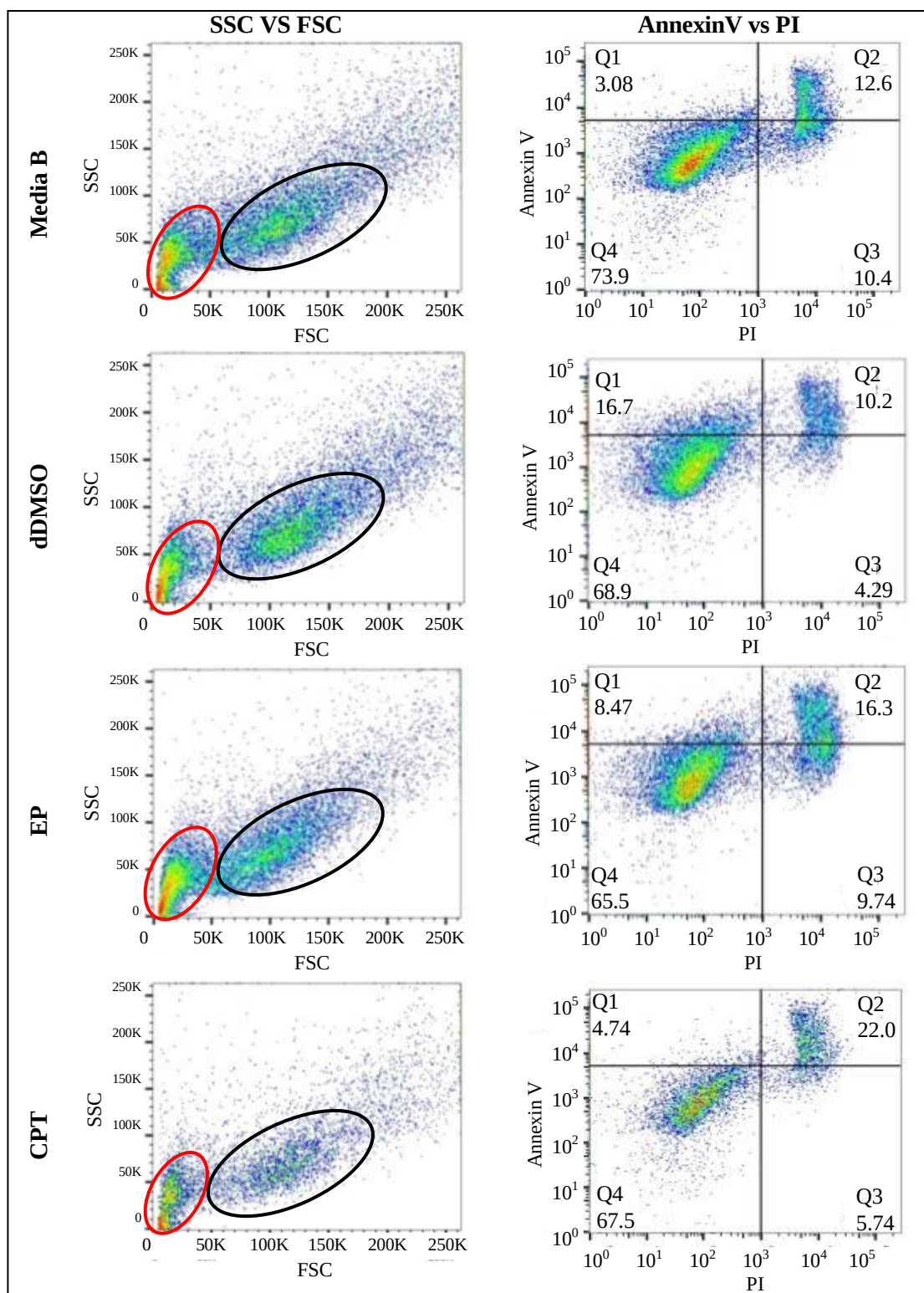


Figure 3.36. Representative scatter graphs showing SCC vs FSC and AnnexinV vs PI of the MCF-7 cells treated with the controls

Control groups: Rows: Media B. dDMSO. EP. Camptothecin (CPT). **Columns:** SCC vs FSC. AnnexinV vs PI. The graphs show two distinct populations, one showing small cells with little granularity (encircled in red) and the second showing larger cells with more granularity (encircled in black). The AnnexinV vs PI graphs are divided into 4 quadrants showing AnnexinV⁺ PI⁻ early apoptotic cells (Q1), AnnexinV⁺ PI⁺ late apoptotic cells (Q2), AnnexinV⁻ PI⁺ necrotic cells (Q3) and AnnexinV⁻ PI⁻ live cells (Q4). In all control treatment groups, the majority of cells are alive (Q4). The highest levels of induced early apoptosis was noted in the DMSO treatment group (dDMSO, Q1) and the highest level of induced late apoptosis was found in the camptothecin treatment group, as expected

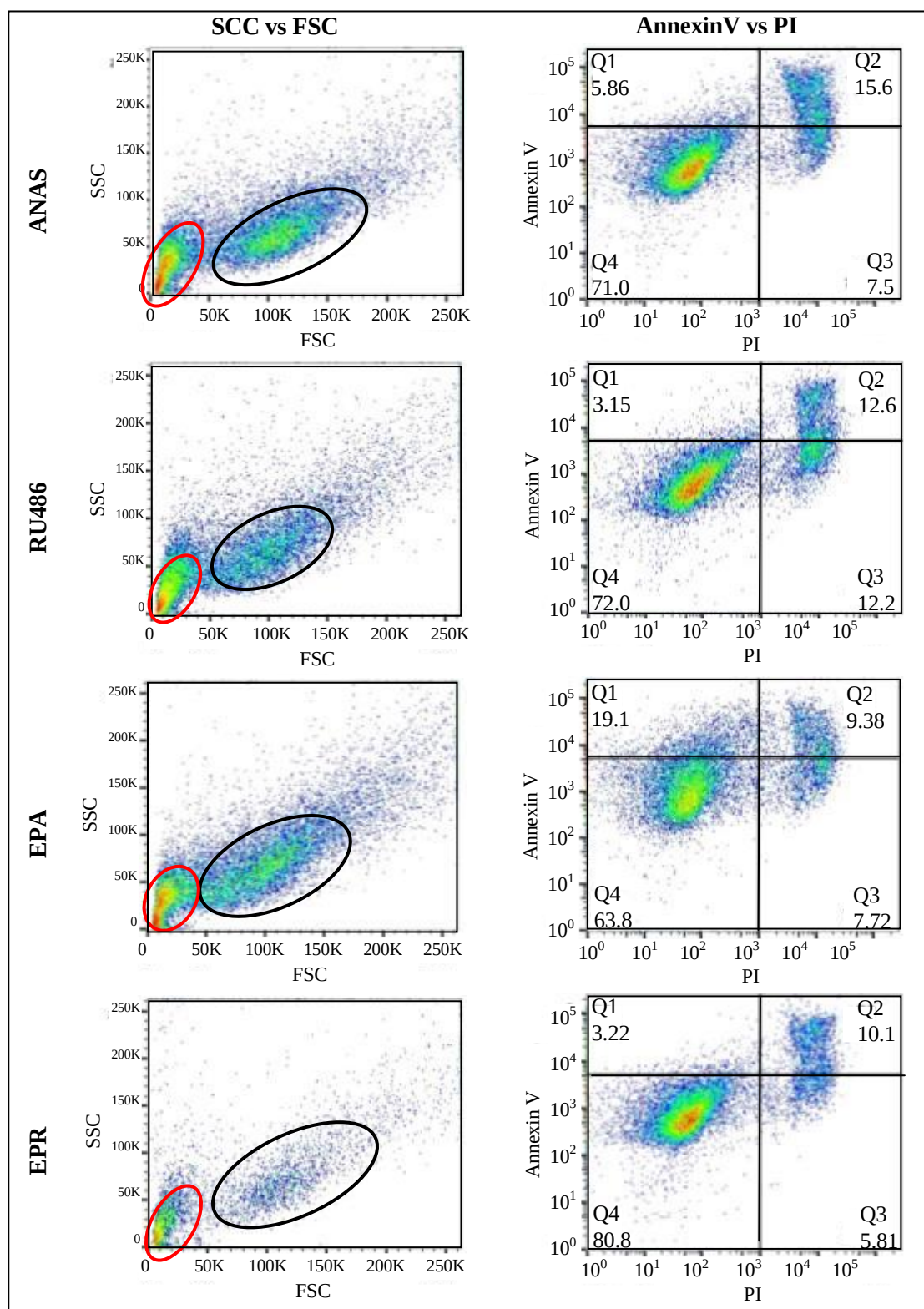


Figure 3.37. Representative scatter graphs showing SCC vs FSC and AnnexinV vs PI of the MCF-7 cells treated with hormone-therapy

Treatment groups: Rows: ANAS. RU486. EPA. EPR. **Columns:** SCC vs FSC. AnnexinV vs PI. The SCC vs FSC graphs show two distinct populations, one showing small cells with little granularity (encircled in red) and the second showing larger cells with more granularity (encircled in black). The AnnexinV vs PI graphs are divided into 4 quadrants showing AnnexinV⁺ PI⁻ early apoptotic cells (Q1), AnnexinV⁺ PI⁺ late apoptotic cells (Q2), AnnexinV⁻ PI⁺ necrotic cells (Q3) and AnnexinV⁻ PI⁻ live cells (Q4).

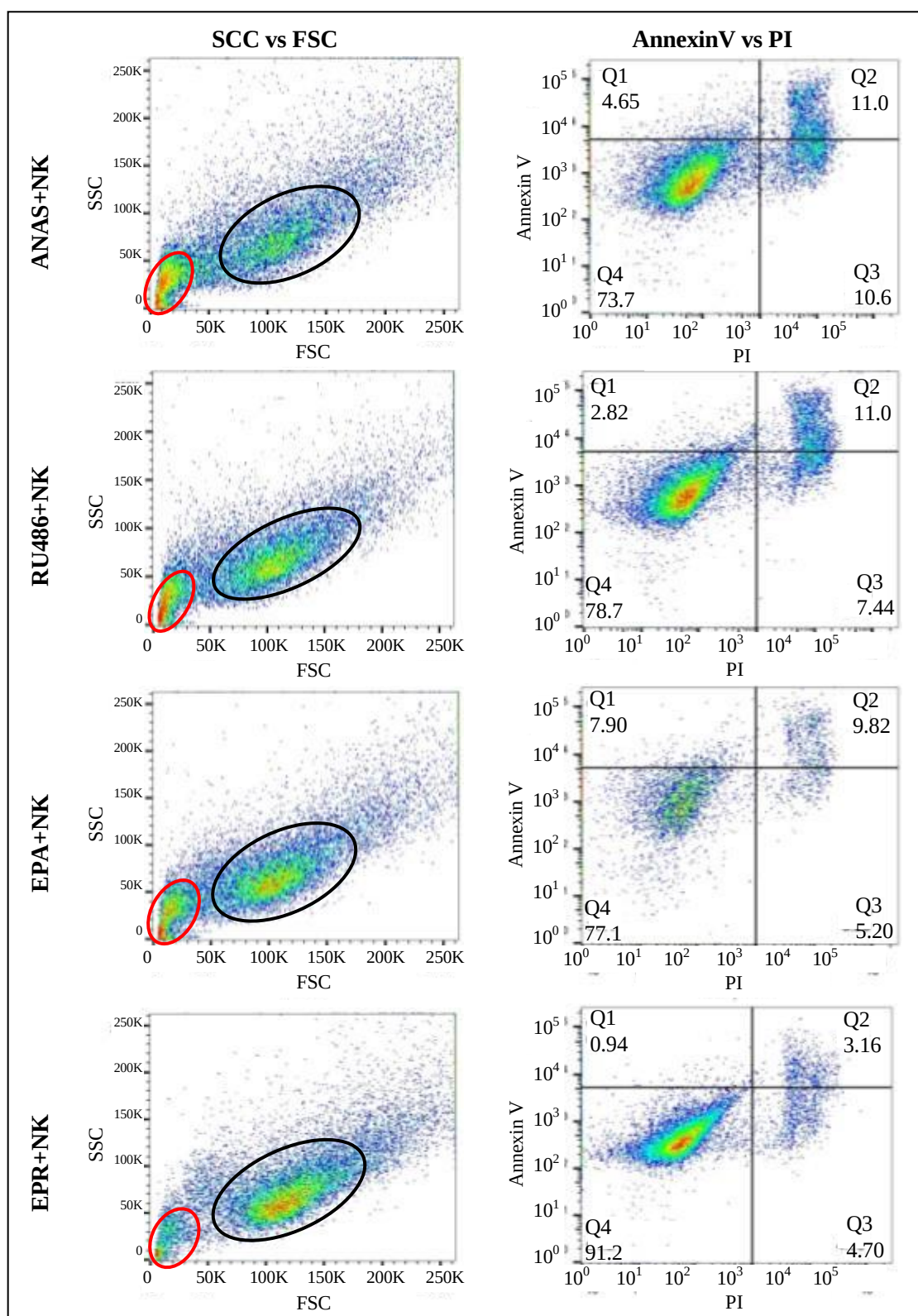


Figure 3.38. Representative scatter graphs showing SCC vs FSC and AnnexinV vs PI of the MCF-7 cells treated with combined hormone-therapy and immunotherapy

Treatment groups: Rows: ANAS+NK. RU486+NK. EPA+NK. EPR+NK. **Columns:** SCC vs FSC. AnnexinV vs PI. The SCC vs FSC graphs show two distinct populations, one showing small cells with little granularity (encircled in red) and the second showing larger cells with more granularity (encircled in black). The AnnexinV vs PI graphs are divided into 4 quadrants showing AnnexinV⁺ PI⁻ early apoptotic cells (Q1), AnnexinV⁺ PI⁺ late apoptotic cells (Q2), AnnexinV⁻ PI⁺ necrotic cells (Q3) and AnnexinV⁻ PI⁻ live cells (Q4).

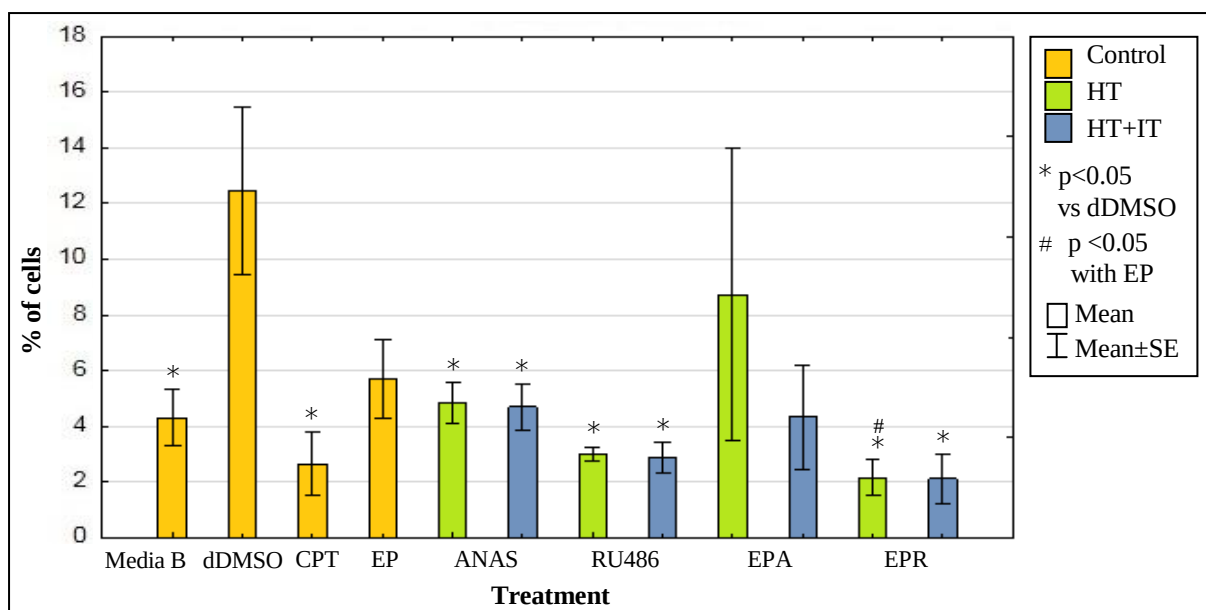


Figure 3.39. A bar graph showing the percentage of cells undergoing early apoptosis (Q1) with the controls and treatment groups

All hormone-therapy treatments, apart from EPA, significantly decreased ($p < 0.05$) the percentage of cells undergoing early apoptosis as compared to the dDMSO control. This decrease ($p < 0.05$) was mimicked in the combined hormone-therapy with immunotherapy treatments, again with the exception of the EPA+NK treatment. When compared to the EP control group, only the EPR treatment caused a significant decrease ($p < 0.05$) in the percentage of cells undergoing early apoptosis. There was also no significant difference detected compared to the drugs alone and between the HT and HT+IT groups regardless of treatment.

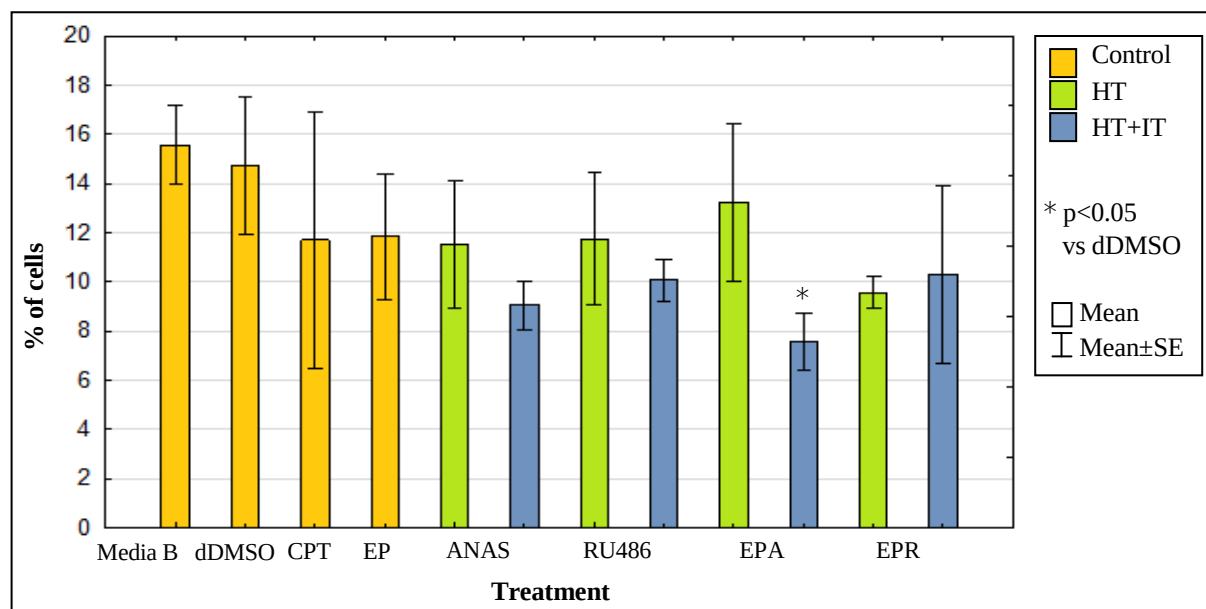


Figure 3.40. A bar graph showing the percentage of cells undergoing late apoptosis (Q2) with the controls and treatment groups

The EPA+NK treatment was the only one that induced a significant difference ($p < 0.05$) when compared to the dDMSO control with late apoptosis. There were no significant differences detected when compared to the EP control, the drugs alone and between the HT and HT+IT groups regardless of treatment.

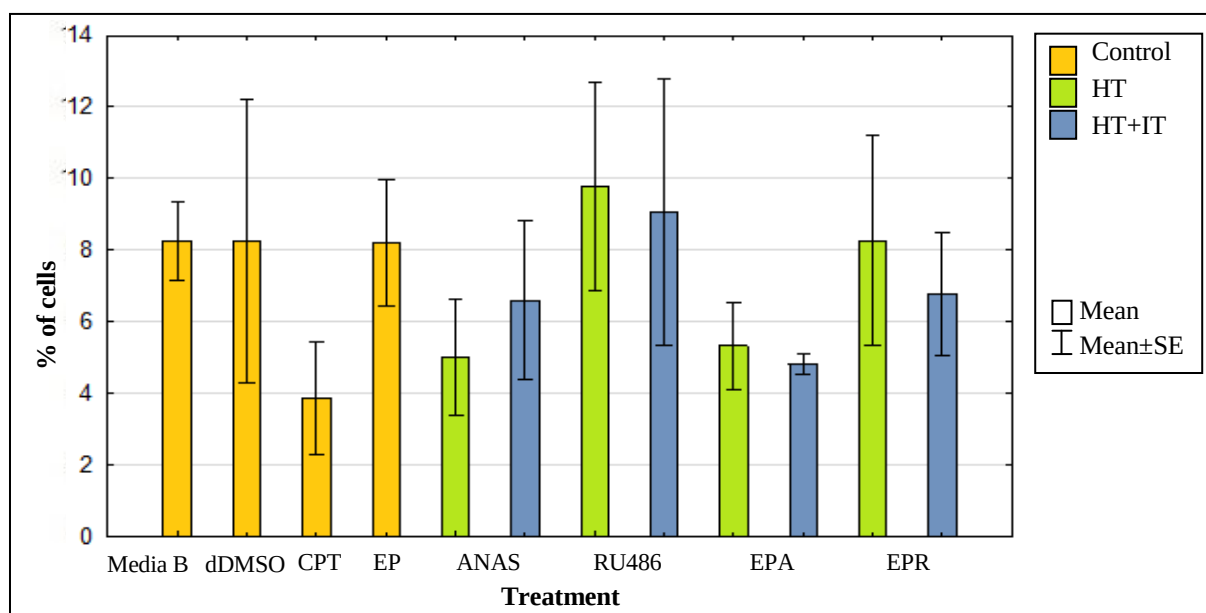


Figure 3.41. A bar graph showing the percentage of cells undergoing necrosis (Q3) with the controls and treatment groups

The was no significant differences ($p > 0.05$) noted when compared to the dDMSO control, EP control, drugs alone and between the HT and HT+IT groups regardless of treatment.

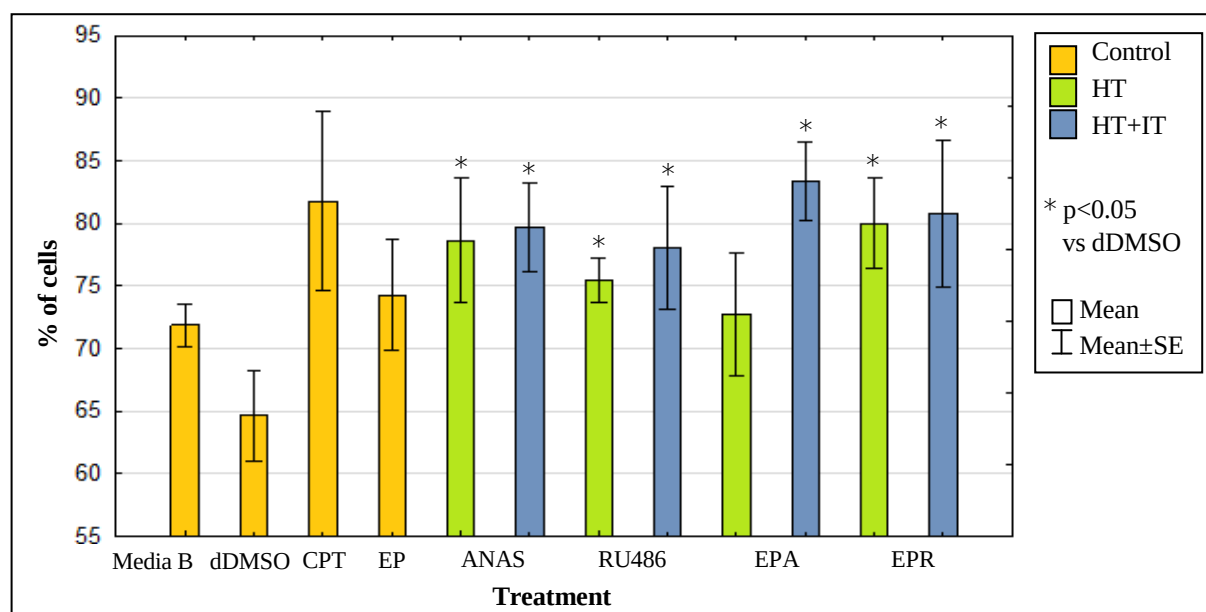


Figure 3.42. A bar graph showing the percentage of live cells (Q4) with the controls and treatment groups

The majority of the treatments (apart from EPA) induced a significantly increased percentage of live cells when compared to the dDMSO control treatment. However, there was also no significant difference when compared to the EP control, the drugs alone or between the HT and HT+IT groups regardless of treatment.

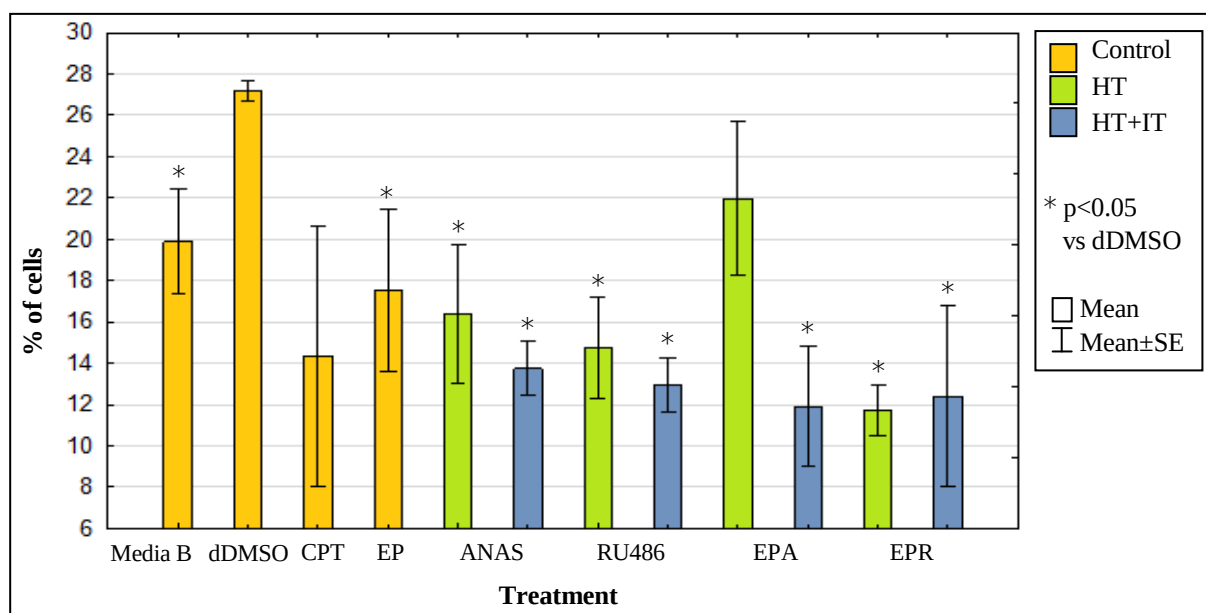


Figure 3.43. A bar graph showing the percentage of cells undergoing total apoptosis (Q1+Q2) with the controls and treatment groups

All treatments (apart from EPA) induced a significantly decreased ($p < 0.05$) percentage of cells undergoing apoptosis as compared to the dDMSO control. However, there was also no significant difference noted when compared to the EP control, the drugs alone or between the HT and HT+IT groups regardless of treatment. This indicates that the addition of NK cell mediation did not induce significant cell death.

DISCUSSION

Biomarker localisation

Traditionally pathologists diagnose ER⁺ and/or PR⁺ tumours by nuclear immunohistochemistry analysis and cytoplasmic staining is disregarded (Welsh *et al.*, 2012). According to the updated St Gallen definition of intrinsic subtypes (Goldhirsch *et al.*, 2013) only nuclear expression is analysed with regards to ER, PR and Ki-67. With ER and PR $\geq 20\%$ indicates positivity and $< 20\%$ indicated negativity. With Ki67 positive nuclei the following classifications are used: low ($< 14\%$), intermediate (14-19%) or high ($\geq 20\%$). However, with regards to HER2, overexpression is regarded as $> 10\%$ of the cells having intense and complete membrane staining (Maisonneuve *et al.*, 2014). However, ER α and PR can exist in the cytoplasm of the cells, where it diverges from its traditional nuclear transcriptional functions and instead interacts with other receptors and cytoplasmic kinases involved in tumour progression (Acconcia and Kumar, 2006; Lange, 2008; Hill *et al.*, 2012; Welsh *et al.*, 2012). This indicates a real need to revise diagnostic definitions to include cytoplasmic localisation of ER and PR biomarkers.

In the present study, quantitative analysis indicated that ER α , PR and MUC1 expression essentially varied between nuclear and cytoplasmic compartments with treatment within the hormone-therapy and immunotherapy groups. Trends observed did not always correspond to the qualitative analysis of the images themselves. This disparity could be due to the subjective nature of qualitative analysis. For example, where images show low ER α /PR intensity with certain treatments as compared to the normal ER α /PR intensity, if only the nucleus is considered, with pure qualitative analysis this could indicate a hormone-negative breast cancer, and a false negative diagnosis. Therefore, the impartial analysis of the images is important and a quantification program like CellProfiler could be used to standardise tumour diagnosis. This program could also allow for additional information about the cells to be analysed, including cell numbers, cell size and comparative cellular compartment sizes (Carpenter *et al.*, 2006).

The Effects of Hormone-Therapy on MCF-7 Cells

Hormone therapy employs the use of selective oestrogen receptor modulators (SERM), selective oestrogen receptor downregulators (SERD), and aromatase inhibitors (AI). SERMs function primarily as ER antagonists, with some agonist properties; SERDs are pure ER antagonists and AIs prevent the aromatase enzyme from converting androgens to oestrogens. (McDonnell and Goldman, 1994; Miller *et al.*, 2005). The hormones of particular interest in hormone-responsive breast cancers are oestrogen and progesterone. In this study, pre-treatment with 17 β -oestradiol and progesterone were conducted to mimic the tumour microenvironment pre-hormone-therapy. Continued treatment with the hormones decreased the expression of all three biomarkers used in this study. The comparative reduction of ER α , as noted under hormone stimulation in this study affirms findings that agonists, specifically oestradiol, are capable of downregulating ER α protein expression indicating transcriptional activity (Pinzone *et al.*, 2004). 17 β -oestradiol is an active form of oestrogen that activates ER causing an increase in DNA synthesis by activating the binding of ER to Src (a tyrosine kinase oncoprotein) which also increases cell survival, proliferation and metastasis (Marino and Ascenzi, 2008). In this study, cytoplasmic ER α expression was noted, albeit reduced under hormonal stimulation, indicating non-genomic functions of this biomarker. Specifically 17 β -oestradiol has been linked to cell growth, cell survival and metastasis by non-genomic regulation of cellular processes by PI3K/Akt activation (PI3K is a lipid kinase that causes the translocation of Akt to the plasma membrane where it has functions in cell proliferation, differentiation and survival (Acconcia and Kumar, 2006)).

Oestrogen in combination with progesterone as a hormone replacement therapy has a significantly higher risk of breast cancer than with oestrogen alone (Fournier *et al.*, 2008), indicating a role for progesterone in breast cancer initiation and progression. In this study, cells under continued hormone stimulation with 17 β -oestradiol and progesterone expressed reduced levels of PR. This result equates to ligand-dependent downregulation of PR which has been noted in other studies, and is related to hyperactivation of the PR (Shen *et al.*, 2001). Progesterone genomic effects include direct binding to progesterone response elements (PREs) in genes (which affects c-myc, fatty acid synthetase and MMTV) or via transcription factors (affects epidermal growth factor receptor, c-fos, p21, IRS-2 and cyclin D1) (Lange, 2008), which increases oncogene expression and thus allows for tumour progression. The intracellular cytoplasmic cascades also affected by progesterone and oestradiol include mitogen-activated

protein kinase (MAPK), PI3K and c-Src kinase (Itoh *et al.*, 2005; Lange, 2008). These cascades cause upregulation of cyclins D1 and E, CDK2 activation, apoptosis mediation as well as an accumulation of cells in the in S-phase of the cell cycle (Itoh *et al.*, 2005; Lange, 2008). This upregulation of oncogenes and oncoproteins in breast cancer cells therefore encourages tumour progression (Leonhardt *et al.*, 2003). The reduction of biomarker expression, particularly ER α and PR, in this study is thus more likely to reflect transcriptional activity of these proteins.

The Effects of Anastrozole on MCF-7 Cells

Anastrozole is an aromatase inhibitor, it binds to the aromatase enzyme and prevents conversion of androgens to oestrogens, thus reducing the available pool of oestrogens for hormone-dependent cancer progression (Miller *et al.*, 2005). In MCF-7 cells, exogenous 17 β -oestradiol has been shown to upregulate 104 genes including *BRCA1*, *cyclin D1*, *IGF-binding protein4*, *pS2*, *BCL2* and down-regulate 109 genes including *BAK1*, *TGF β* , *ERBB2*, *ERBB3* and *Era* (Itoh *et al.*, 2005). All these genes are implicated in tumour progression, proliferation, immune evasion and anti-apoptosis in cancer cells. Of the genes upregulated and downregulated by 17 β -oestradiol in the MCF-7 cell line, Anastrozole treatment downregulated 75% of the upregulated genes, and upregulated 78.9% of the downregulated genes (Itoh *et al.*, 2005). This shows a reversibility of the oestrogen effects on hormone-dependent breast cancer cells.

Anastrozole treatment (at 1 μ mol/L for 1 week) of MCF-7aro cells (cells transfected with the aromatase gene) has been shown to downregulate ER α gene expression (Itoh *et al.*, 2005). Other studies conversely showed that MCF-7 cells treated with a combination of 10 nM 17 β -oestradiol and 100 nM Anastrozole for 48 hours marginally increase ER α expression (Smollich *et al.*, 2009). In our laboratory, it has previously been found that 72 hour Anastrozole treatment caused an increase in nuclear and cytoplasmic ER α expression (Gil *et al.*, 2013). However, in the present study, Anastrozole treatment following a hormone pre-treatment (both at 1 μ M) to simulate *in vivo* conditions induced a decreased ER α nuclear and higher ER α cytoplasmic expression, as well as a decreased PR expression. The addition of simultaneous hormone treatment further reduced ER α and PR expression.

The variance in the aforementioned results is perhaps due to the indirect effects of progesterone, in the current study, on ER α expression (McDonnell and Goldman, 1994; Lange,

2008). In MCF-7 cells, PR-A has been found to regulate ER function (McDonnell and Goldman, 1994) indicating that hormone therapies that target only oestrogen and the ER may not be as effective as a combination against both oestrogen/ER and progesterone/PR. While oestrogen induces proliferation, progesterone seems to be a contrary steroid producing both proliferative as well as inhibitory responses in MCF-7 cells as they seem to require the presence of other receptors such as ER α and EGFR (Lange, 2008). PRs also associate with co-regulators, they can also upregulate genes without PREs through other factors and bind directly to proteins in the cytoplasm (Lange, 2008).

In the current study, Anastrozole treatment induced a reduction in PR expression, more so when additional hormonal stimuli were present, indicating a particular role for hormonal regulation of the PR. Clinically, patients with ER+/PR+ breast cancers undergoing Anastrozole treatment showed that a comparatively higher PR expression, but not ER expression, was linked with an increased time to treatment failure and increased overall survival (Anderson *et al.*, 2011). Further *in vitro* studies would need to be performed to determine if long-term treatment with Anastrozole following hormone pre-treatment would correspond to clinical results.

Interestingly, Gil *et al.* (2013) showed that deprivation of hormones from culture media resulted in upregulated ER α expression, as a possible compensatory mechanism for low hormonal stimuli. Clinically, downregulation of ER α is associated with increased recurrence and metastasis (Dhasarathy *et al.*, 2007; Aktas *et al.*, 2011). Thus, in the current study, following Anastrozole treatment the reduction in PR and ER α may in fact be indicative of an increased invasive profile. Morphologically, cells exhibiting a more mesenchymal phenotype (small, elongated cells) are regarded as being more invasive. The MCF-7 cells in this study presented a heterogeneous morphology, displaying cells associated with a more mesenchymal phenotype, as well as the more typical polygonal cells, indicative of more differentiated phenotypes (Dhasarathy *et al.*, 2007). Invasive phenotypes are characterised as being highly proliferative. Ki67 is clinically used as a marker of proliferation (Scholzen and Gerdes, 2000). However, the present study showed no significant change in proliferation (Ki67) with any hormone-therapy treatment regardless of simultaneous hormone treatment.

Moreover, while studies implicate progesterone alone or in combination with 17 β -oestradiol in increasing metastatic potential of breast cancer cells (Faivre *et al.*, 2005; Fu *et al.*, 2008), in the current study, treatment with 17 β -oestradiol and progesterone alone did not significantly

increase the proliferative potential of MCF-7 cells. These hormones instigate upregulation of certain oncogenes which allows for increased proliferation, decreased apoptosis and metastatic progression of these cells (Faivre *et al.* 2005, Fu *et al.*, 2008). However, proliferation is an active event and only part of the invasive process. Our results hint at the production of a pre-metastatic profile which may be mediated by cytoplasmic expression of ER α and PR.

While clinical assessment of ER/PR expression considers only nuclear presentation, PR and ER function in two major ways, genomically causing transcription of target oncogenes and non-genomically stimulating intracellular signalling cascades (Wen *et al.*, 1994; Zhang and Trudeau, 2006). Cytoplasmic staining is associated with non-genomic effects of these receptors, and thus may contribute to tumour progression independently of transcriptional events. Cytoplasmic ER α , for example, is implicated in the induction of matrix metalloproteinase function which promotes tumour invasion (Björnström and Sjöberg, 2004). That Anastrozole treatment without additional hormonal influences, increased cytoplasmic ER α expression in this study, is thus of concern, considering that such localisation of this marker is not considered in clinical diagnosis.

Additionally, Anastrozole treatment in the present study induced a significant increase in MUC1 expression. This corresponds to previous results from our lab performed with no hormone pre-treatment (Gil *et al.*, 2013), which indicates that the hormone pre-treatment had no definitive effect with regards to MUC1 expression. MUC1 is upregulated in the majority of breast cancers and has been implicated in interacting directly with ER α , stimulating co-activator binding as well as ER α -mediated transcription (Wei *et al.*, 2006). Thus the substantial increase in MUC1 intensity, in this study, may be a compensatory mechanism induced by MCF-7 cells in response to Anastrozole treatment and the concomitant reduction in ER α expression. This increase in MUC1 is postulated to occur as a non-ER α -mediated transcription under the influence of other factors. Importantly, the cytoplasmic domain of MUC1 can undergo nuclear translocation where it may induce ER α transcription (Wei *et al.*, 2006; Khodarev *et al.*, 2009). It is proposed that the increase in MUC1 as noted in this study is thus a compensatory mechanism that, given time, would allow for the replenishment of ER α . Long term *in vitro* studies are needed to verify this postulation. Notably, the present study shows that simultaneous treatment with hormones and Anastrozole decreased MUC1 expression. This contrasted with qualitative analysis which, when co-localised, revealed that MUC1 dominated under hormonal stimulation. Correlation analysis of MUC1 and ER α , further showed no

definitive association between the biomarkers; however, as seen visually, hormonal stimulation resulted in a stronger, yet low correlation being attained. Interestingly, progesterone binding to PR-A inhibits ER transcriptional activity (Wen *et al.*, 1994) which includes the *MUC1* gene (Zaretsky *et al.*, 2006) and may thus be responsible for low correlation.

MUC1 interaction with PR has been indicated in endometrial cell lines, which are also hormone-dependent, and ER α in breast cancer cells (Horne *et al.*, 2006; Wei *et al.*, 2006; Dharamaraj *et al.*, 2010). In this study, treatment with Anastrozole induced nuclear and cytoplasmic MUC1 dominance over PR. It was further determined that under Anastrozole treatment MUC1 and PR strongly correlated. However, this correlation was dependent on cytoplasmic localisation, again illustrating the importance of considering staining in this compartment. Interestingly, MUC1 no longer dominated PR expression with the addition of continuous hormone treatment, with the correlation between markers fairly strong in the cytoplasm only. This indicates hormonal regulation of MUC1 (Horne *et al.*, 2006; Wei *et al.*, 2006; Dharamaraj *et al.*, 2010). Our results confirm clinical data that show breast tumour cytoplasmic MUC1 expression is significantly associated with PR expression (van der Vegt *et al.*, 2007).

MUC1 can also translocate to the mitochondrial outer membrane, inhibiting the release of apoptotic proteins (Kufe, 2009). Previous studies have shown that Anastrozole treatment downregulated the anti-apoptotic Bcl-2 gene (Itoh *et al.*, 2005), which would allow for an increase in apoptosis. In the present study, we assessed phosphatidylserine expression as an early marker of apoptosis, with additional nuclear propidium iodide staining indicating later stages of cell death. Anastrozole induced a significant decrease in cells undergoing apoptosis. This divergence with laboratory data (Itoh *et al.*, 2005), conversely supports clinical data which shows that Anastrozole treatment is not linked to reduced tumour size (Cuzick *et al.*, 2014). While our results could be due to the pre-treatment of the cells with hormones, indicating that the hormone environment plays a critical role with regards to protection against apoptosis, with concurrent hormone treatment there was no significant difference in apoptotic cell numbers. Further studies are necessary to elucidate the role of Anastrozole in apoptosis induction. We further propose that since cytoplasmic MUC1 may be involved in limiting apoptosis that the effects of Anastrozole may have been impeded by this protein. This postulation requires verification.

The Effects of RU486 on MCF-7 Cells

RU486 is a progesterone antagonist usually used as ‘the morning after’ pill to prevent pregnancy. It is not commonly used as a cancer hormone-therapy treatment, as progesterone effects in hormone-dependent cancer are still not fully understood or agreed upon. However, as a progesterone antagonist, it binds to the LBD of the PR inhibiting progesterone-mediated activity (Vegeto *et al.*, 1992). Therefore, in the present study, it was interesting to see the effects of RU486 on biomarkers in hormone-dependent breast cancer.

In our lab, it has been previously found that 72 hour RU486 treatment caused a significant increase in ER α and MUC1 expression in the nucleus and cytoplasm (Gil *et al.*, 2013). The present study confirms the increased ER α expression, particularly in the cytoplasm. However, the hormone pre-treatment conversely affected MUC1 expression, causing a significant decrease compared to results found by Gil *et al.* (2013). In this study RU486 treatment induced a significant decrease in PR expression as well. This correlates to the clinical study conducted by Horwitz (1992) whereby 3 week RU486 treatment decreased tumour PR expression. However, in the present study, the simultaneous treatment with RU486 and hormones caused an increase in PR expression while concomitantly reducing MUC1 and ER α expression. The results of this study indicate that PR expression was affected by hormonal stimuli; however, PR mediated transcriptional activity is not fully dependent on progesterone alone (Lange, 2008). As previously discussed, a higher PR expression correlates to an increase in the time to treatment failure and thus overall survival (Anderson *et al.*, 2011). Therefore, the short-term treatment in this study with simultaneous RU486 and hormones (indicative of an *in vivo* environment) would indicate a potential positive impact on patient survival. However, cognisance must be given that cytoplasmic PR as noted in this study, may mediate signal transduction pathways independently of transcriptional events, which include promoting cells through the cell cycle and thus promoting tumour progression (Leonhardt *et al.*, 2003).

However, it must be noted that RU486 treatment in this study had no significant effect on expression of the proliferative marker Ki67, regardless of additional hormonal stimulation. These results contradict reports of RU486 inhibition of hormone-dependent breast cancer cell proliferation (Horwitz *et al.* 1992). This inhibition was shown to be mediated by PR expression, and in our study, PR expression in RU486 treated cells alone, was decreased. With the addition of hormones and despite the increase in PR expression, RU486 was still unable to inhibit proliferation. Progestins have been shown to stimulate proliferation, therefore the

combination of hormones with RU486, may have abrogated the inhibitory effect of the drug (Moore *et al.*, 2006). Further analysis must be undertaken to identify the mechanism responsible for the stasis seen in the current study.

As previously mentioned, MUC1 gene expression has been linked to progesterone-PR mediated transcription in endometrial cell lines (Horne *et al.*, 2006; Dharmaraj *et al.*, 2010). The results obtained in the current study indicate that MCF-7 cells are also influenced by progesterone and the PR. RU486 treatment induced a strong correlation between PR and MUC1, and ER α and MUC1, regardless of cellular localisation. With simultaneous treatment with hormones, the strong correlation between PR and MUC1 expression was maintained in the nucleus only. Importantly, RU486 has PR agonist effects acting specifically on the PR-B isoform (Hill *et al.*, 2012). The PR antibody used in this study did not differentiate between isoforms therefore any agonist effects could not be isolated. That RU486 has PR-independent actions that have yet to be fully understood (Tieszen *et al.*, 2011, Chen *et al.*, 2014), and may explain the effects seen on ER α expression and MUC1 expression.

RU486 has been shown in short-term clinical studies to inhibit mammary tumour growth without inducing a reduction in tumour size (Horwitz, 1992). In the present study, RU486 with or without simultaneous hormone treatment induced a significant decrease in the number of apoptotic cells. However, the additional hormone stimulation did result in a lower number of cells undergoing apoptosis, reflecting observations that progesterone and oestrogens inhibit apoptosis in breast cancer cells (Moore *et al.*, 2006). Furthermore, RU486 has weak oestrogenic activity (Jeng *et al.*, 1993), the effects of which may abrogate the antagonistic function of the hormone-therapy drug. Further experimentation is required to fully map the effects of RU486 on cell death.

The Effect of Immunotherapy on MCF-7 Cells

Cancer immunotherapy is the manipulation of the immune system for the treatment of cancers. These include monoclonal antibody therapy, non-specific immunomodulation, vaccines and adoptive cell transfer (Rosenberg *et al.*, 2008). Adoptive cell transfer (ACT) is the identification of anti-tumour lymphocytes *ex vivo* which are then cultured and expanded and used to treat patients (Rosenberg *et al.*, 2008). Considerable research has been performed with T-cells and fewer with NK cells. NK cells are not typically found in breast tumours; however, it has been established that patients infused with NK cells can undergo remission (Fujisaki *et al.*, 2010).

NK cells are part of the innate immune system and they differentiate between cancerous cells and healthy cells by detecting the balance between the activating and inhibitory signals they receive (Sutlu and Alici, 2009). The activating receptors on NK cells include NKp46 (Sutlu and Alici, 2009) which in the current study was used to positively select the NK cells employing a magnetic cell sorting technique. This marker is found on the majority of NK cells and a high concentration is associated with a high cytotoxic capacity (Miller, 2001). The magnetically sorted NK cells were activated with IL-2, which induces proliferation and differentiation, and PHA, a mitogen (Domaica *et al.*, 2009). Flow cytometric analysis of NK cell phenotype was conducted in our laboratory after magnetic sorting was performed (Augustine *et al.*, 2015). This indicated 4 populations based on the CD56 and NKp46 markers. However, after activation with IL-2 and PHA only 2 populations were observed and both were CD56^{dim} NKp46^{bright}, CD56^{dim}NKp46^{dim} (Augustine *et al.*, 2015). It has been shown that CD56^{dim} NK cells express the oestrogen receptor isoforms, ER α and ER β (Curran *et al.*, 2001); and the progesterone receptor isoforms PR-A and PR-B (Arruvito *et al.*, 2008). These cells may thus be susceptible to hormones and hormone-therapy. This indicates that hormones as well as hormone-therapy drugs would affect NK cell function.

In the present study, NK cells were co-cultured with MCF-7 cells during hormone and hormone-therapy treatments to simulate ACT. All the treatments induced a significant decrease in PR expression. However under NK cell mediation, the addition of hormones to both RU486 and Anastrozole treatment induced a further reduction in cytoplasmic PR expression while concomitantly increasing nuclear PR expression. These results correlate with observations that cytoplasmic PR isoforms decrease in response to 24hr RU486 treatment, with nuclear PR expression remaining elevated for a longer duration (Wei *et al.*, 1997). Moreover, while the

present study did not distinguish between the PR isoforms, high levels of PR-A are implicated in more aggressive tumour behaviour compared to high expression of PR-B (Jacobsen *et al.*, 2005). It is thus necessary to conduct further experiments using the different isoforms to more accurately assess implications of alterations in PR expression. However, the data suggests that NK mediation together with hormone-therapy, under hormone stimulation, differentially affects cellular compartments, which is important when considering the genomic and non-genomic effects of PR.

Under immune mediation MUC1 and PR showed a strong nuclear correlation, with both Anastrozole and RU486 treatment, with PR dominating the nucleus. With the addition of hormones, correlation between the biomarkers reduced, with slight dominance of PR in the cytoplasm. These results again implicate PR genomic transcription of factors involved in tumour progression (Wen *et al.*, 1994; Zhang and Trudeau, 2006). While tumour progression also takes into account cellular proliferation, in the current study, immunotherapy decreased the presentation of the Ki67 proliferative marker in all hormone-therapy treatment groups; however this result was not significant and thus impedance of proliferation cannot be affirmed.

NK cell mediation also had a strong influence on the relationship between MUC1 and ER α , affirming the association between these two markers as previously mentioned (Wei *et al.*, 2006). MCF-7 cells treated with combined immunotherapy and Anastrozole or RU486 hormone-therapy induced a significant increase in cytoplasmic ER α expression and decrease in nuclear ER α expression. With additional simultaneous hormone treatment, there was a comparative increase in expression in both compartments. As previously mentioned, downregulation of ER α is associated with increased recurrence and metastasis in a clinical setting (Dhasarathy *et al.*, 2007; Aktas *et al.*, 2011); thus this result may indicate that this three-pronged approach shows the efficacy of NK cell mediated ACT. However, we must consider that ER α can in turn mediate transcription of MUC1 (Wei *et al.*, 2006). The results may thus conversely indicate a shift to MCF-7 cell defence against NK cell cytotoxic capacity, since MUC1 facilitates evasion of the immune response (Yawata *et al.*, 2008).

MUC1 expression in combined hormone-therapy and immunotherapy mimic the PR results, with a decrease in expression noted with both Anastrozole and RU486 treatment. However, the addition of simultaneous hormone treatment, increased MUC1 expression, indicating that the hormones partially abrogated the effects of the combined hormone-therapy and immunotherapy. 17 β -oestradiol has been shown to significantly decrease NK cell activity in

NK cells isolated from mice (Curran *et al.*, 2001). Added to which is that MUC1 has a large impact on protein-protein interaction in cancer cells and can influence cancer progression. These results bear out observations that MUC1 expression may be induced in MCF-7 cells to limit NK cell function.

NK cells function primarily in cytotoxicity, and it was thus expected that activated NK cells would induce apoptosis in MCF-7 cells. However, in this study, using Anastrozole or RU486 significantly decreased the induction of apoptosis even under hormone mediation. Clinical studies have shown that Anastrozole treatment of postmenopausal ER⁺ breast cancer patients causes an increase in total white blood cell count but no change in lymphocyte count specifically (Ray and Ficek, 2012). The treatment also caused increased cytokines, IFN γ and IL-12, and decreased IL-4 and IL-10 levels (Ray and Ficek, 2012). This indicates that NK cell numbers may not be as important as the functionality of NK cells. In the present study, a low NK cell concentration was used to treat the MCF-7 to mimic the tumour environment more closely; however, the concentration used was still higher than found naturally in tumours. There is no information about the effect of NK cells on PR expression in breast cancer. However, as previously mentioned, CD56^{dim} NK cells express the oestrogen receptor isoforms, ER α and ER β (Curran *et al.*, 2001); and the progesterone receptor isoforms PR-A and PR-B (Arruvito *et al.*, 2008), and it is thus possible that the hormone therapies may have indeed directly affected NK cell function.

The Difference between Hormone-Therapy and Combined Therapy

In the current study, NK cell presence induced a decrease in MUC1 expression with Anastrozole and RU486 treatment, compared to the hormone-therapy alone. This decrease in MUC1 could potentially make the MCF-7 cells more susceptible to NK cell-mediated cytotoxic action; however, this was not evident as no increase in apoptotic cells was noted. However, it must be noted that MUC1 can be secreted – this form of MUC1 would have been undetectable in this study – and has been shown to inhibit cytotoxic lymphocyte function (Zhang *et al.*, 1997). With the addition of hormonal stimuli to the immunotherapy procedure, MUC1 effectively increased under both Anastrozole and RU486 treatment. This indicates a mechanism of hormonal regulation of MUC1. It has been documented that increased MUC1 inhibits NK cell mediated lysis in NK-sensitive K562 cells with a radiolabelling lysis assay at 4 hours

(Zhang *et al.*, 1997). However, when K562 cells were co-incubated with MUC1, MUC1 bound to NK cells effectively inhibiting NK cell-mediated apoptosis (Zhang *et al.*, 1997). Although Cooley *et al.* (1999) found that MCF-7 cells are not inherently resistant to NK cells, other studies have reported otherwise (Burns *et al.*, 2003). Furthermore, IL-2 activation of NK cells has been seen to reduce the effect of this resistance (Burns *et al.*, 2003; Sutlu and Alici, 2009). Therefore, MUC1 mediated inhibition of NK cell function could be an explanation for the decreased apoptotic activity seen in the current study, notwithstanding that MUC1 as previously mentioned can inhibit the release of apoptotic proteins from the mitochondria (Kufe, 2009).

Oestrogen also has a function in inhibiting apoptosis in the MCF-7 cell line by increasing anti-apoptotic Bcl-2 protein expression and decreasing pro-apoptotic caspase-3 activation (Kandouz *et al.*, 1996). In breast cancer, progesterone increases anti-apoptotic bcl-xL expression and decreases apoptotic bcl-2 expression, promoting cell survival by decreasing apoptosis (Moore *et al.*, 2000), and thereby promoting tumour progression. Further evidence for the amelioration of apoptosis induction by hormonal intervention is indicated by 1 μ M oestrogen treatment reducing NK cell cytotoxic activity in mice, thereby increasing spontaneous tumour generation (Ferguson and McDonald, 1985). In the current study, the combined therapeutic modality, whether using RU486 or Anastrozole treatment, in a hormone environment increased ER α expression compared to hormone-therapy alone. Conversely, without the hormones, ER α expression was decreased. This highlights that NK cell function is somehow impeded in our culture system.

NK cells have been found, both in *in vitro* and mouse *in vivo* studies to be able to target tumour cells (Levy *et al.*, 2011). Despite an increase in NK stimulatory molecules found in tumour cells, NK cells are rarely found in the tumour (Levy *et al.*, 2011; Leibovici *et al.*, 2011). However, tumour infiltrating NK cells have abnormal receptor expressions indicating the interaction between tumour cells and NK cells may result in loss of receptor expression for immune evasion (Levy *et al.*, 2011). In breast cancer, the NK cell activating receptors shown to be decreased are NKp30, NKG2D, DNAM-1 and CD-16, while the inhibitory receptors shown to be increased are NKG2A and CD-27 (Mamesier *et al.*, 2011). These alterations decreased the cytotoxic capacity of NK cells via degranulation (CD107). However, it did not affect their cytokine production of IFN- γ and TNF- α . Thus, it has been suggested that cytokine-mediated activation of NK cells may overcome this barrier either *in vivo* or *ex vivo* (Levy *et al.*, 2011). A

clinical trial administrating IL-2 activated NK cells and IL-2 to patients with tumours showed an increase in NK cell mediated cytotoxicity in lymphoma, melanoma and renal cancers (Geller *et al.*, 2011). The cytokine IL-2 has been implicated in tumour shrinkage, increased NK cell cytokine production as well as increased NK cell proliferation (Lechner *et al.*, 2011). Other cytokines that have also been found to have various anti-cancer effects include TNF- α , IFNs and IL-12 (Leibovici *et al.*, 2011). However, these cytokines can also induce tumour progression. In the present study, no significant effect was found with regards to NK cell cytotoxic activity on the MCF-7 cells although IL-2 stimulation was used. A further study would be to assess cytokine production by NK cells when used as a treatment in conjunction with hormone-therapy, as well as assess their cytotoxic capacity and effects on receptor expression in breast cancer cells.

In the current study, combined therapy induced a significant decrease in PR expression as compared to hormone-therapy alone, regardless of hormone mediation. The PR has been linked to mature peripheral NK cells (CD56^{dim}CD16⁺KIR⁺) only and when activated by progesterone causes caspase-dependent cell death and suppresses cytokine secretion (particularly IL-12-induced IFN- γ secretion) (Arruvito *et al.*, 2008). These effects are inhibited by RU486. However, the CD56^{bright} NK cells are not affected in this way (Arruvito *et al.*, 2008). In the present study, there was a significant decrease in the number of cells undergoing apoptosis when treated with the NK cells regardless of the hormone-therapy treatment. This could be explained by the low ratio of NK cells to MCF-7 cells used in this study, although this was done to mimic the low numbers of NK cells within a tumour (Mamessier *et al.*, 2011). A future study would be to determine if the NK cells have a change in their receptor status and/or if there is hormone induced apoptosis of the NK cells themselves.

Further Studies and Limitations

There have been many advances in breast cancer treatments, yet despite this, the majority of patients rarely survive longer than 5 years (Youlten *et al.*, 2012). Breast cancer immunotherapy, including NK cell ACT, has also proved ineffective so far (Rosenberg *et al.*, 2008; Geller *et al.*, 2011). These ineffective treatment modalities suggest a more complex tumour interaction with the tumour microenvironment. Therefore, a more complex strategy is needed to combat the tumour evasiveness of the current treatments. In the current study, the impact of a combination hormone-therapy and NK cell immunotherapy was investigated in MCF-7 cells. The results indicate that the combined treatment modality reduced biomarker expression of the MCF-7 cells when compared to hormone-therapy alone.

A restriction of this study was the yield of NK cells by the volume of blood permitted per volunteer (Human Ethics Clearance M081036). It would be advisable to increase the volume of blood obtained for isolation of lymphocytes, ethical issues permitting, to allow for the establishment of increased MCF-7:NK cell ratios. NK cells also express ER α and ER β (Curran *et al.*, 2001) and PR-A and PR-B (Arruvito *et al.*, 2008) implicating hormones in immune control. Further studies would involve assessing ER and PR expressions in NK cell populations after hormone-therapy. In the current study we looked at apoptosis in the MCF-7 cells after hormone-therapy and NK cell immunotherapy. A limitation noted was that camptothecin treatment did not induce a significant increase in apoptosis to act as a definitive positive apoptosis control; therefore, another apoptotic inducer for MCF-7 cells would have to be found. Staurosporine (1 μ M) has been found to significantly increase phosphatidylserine translocation in MCF-7 cells at 4 hours (Mooney *et al.*, 2002) and its use is thus proposed for the induction of early apoptosis. A further study would be to assess cytokine production by NK cells, as well as assess their cytotoxic ability and effects on receptor expression in breast cancer cells.

The current study investigated short-term treatment of MCF-7 cells with hormone-therapy (72 hours) and immunotherapy (4 hours). A further study is needed to determine if long-term treatment with hormone-therapy and immunotherapy would affect the results. This long-term treatment would correspond to clinical treatment of breast cancer with hormone-therapy. Adding onto this, an investigation regarding these therapies in a 3D culture system would mimic tumour morphology more closely than in a 2D culture system (Kim, 2005; Augustine *et al.*, 2015). This would produce more clinically relevant and accurate results.

Importantly, we have shown that the selection of appropriate controls is essential to the development of experimental protocols and the interpretation of results. In this study, it was determined that the three levels of control treatments used showed considerable variation in relation to the expression of PR, ER α and MUC1. Media B was the cell culture medium used to induce cell cycle synchronisation. The dDMSO was viewed as a more meaningful control, given that it served as the diluent control for all hormone-therapy drugs and the hormones themselves, while the EP control served as a hormone control for 17 β -oestradiol and progesterone action alone. In an animal model *in vivo* the hormones and drugs can be dissolved in peanut oil (Watts *et al.*, 1994; Asselin-Labat *et al.*, 2010); which in itself remains an additional variable. While the use of DMSO as a diluent for the hormone-therapy drugs and the hormones may be regarded as a limitation of this study, we have identified that this diluent affected biomarker expression as well as the number of apoptotic cells. Comparisons were thus performed with regards to DMSO and not the Media B control to account for this limitation. It must be noted that hormones, as steroids, would not be dissolvable in PBS. Ethanol could be employed as a diluent; however it too would have most likely has its own effects on MCF-7 cells. With regards to *in vitro* treatment of cells, some studies used DMSO as a vehicle control with regards to 17 β -oestradiol (Bursch *et al.*, 1996; Itoh *et al.*, 2005; Wessler *et al.*, 2006; Sukocheva *et al.*, 2009), progesterone (Sukocheva *et al.*, 2009), anastrozole (Itoh *et al.*, 2005; Gil *et al.*, 2013) and RU486 (Sukocheva *et al.*, 2009; Gil *et al.*, 2013). Ethanol is also used as a vehicle control with 17 β -oestradiol (Métivier *et al.*, 2003; Wu *et al.*, 2004; Sonne-Hansen and Lykkesfeldt, 2005; Brayman *et al.*, 2006; Moore *et al.*, 2006; Maia *et al.*, 2008), progesterone (Ahola *et al.*, 2002; Richer *et al.*, 2002; Brayman *et al.*, 2006; Moore *et al.*, 2006; Dharmaraj *et al.*, 2010), anastrozole (Smollich *et al.*, 2009) and RU486 (Ahola *et al.*, 2002; Wu *et al.*, 2004; Brayman *et al.*, 2006; Moore *et al.*, 2006). Although these studies compare their treatments to the vehicle control they do not indicate whether the vehicle they used actually had an effect on the cells. Other studies using 17 β -oestradiol, progesterone, anastrozole or RU486 do not indicate a vehicle control and compare their results to untreated cells (Cho *et al.*, 2004; Wei *et al.*, 2006). This study highlights the necessity of examining expression patterns induced by diluents alone.

Another limitation is the PR antibody utilized in this study, rabbit anti-human PR (Abcam ab97801). It is a general PR antibody, binding to PR regardless of isoform. The different PR isoforms have been implicated in differing responses in breast cancer and a high ratio of PR-A/PR-B has been associated with a poorer outcome in patients undergoing hormone-therapy

(Hopp *et al.*, 2004). Therefore, another study would be to assess these isoforms with regards to the treatment modalities in this study. This would provide additional information regarding these treatment mechanisms in breast cancer. It has been noted that oestrogen alters breast cancer gene profiles, with Anastrozole partially abrogating these alterations (Itoh *et al.*, 2005). Progesterone also alters breast cancer gene expression, mainly via PR-B (Richer *et al.*, 2002). Therefore, a comprehensive gene expression analysis with the treatment modalities outlined in this study would be valuable.

Lastly, it is proposed that Anastrozole and RU486 treatment, together targeting both hormones and hormone receptors would produce a unique response. This dual hormone-therapy approach may reduce the ability of breast cancer cells to minimise the effects of one drug by the cross-talk of the other hormone and hormone receptor.

CONCLUSION

The quantification of biomarker expressions to classify breast cancers is a useful tool, and with this quantification a standardisation for diagnosis is beneficial. The results of this study indicate that consideration of both cytoplasmic and nuclear staining is important to understanding tumour progression and treatment, where much remains unknown. Increased nuclear expression would indicate increased genomic effects while increased cytoplasmic expression would indicate increased activation of signalling pathways important in tumour progression. The use of a hormone pre-treatment to simulate the *in vivo* hormone environment is also an important step for hormone-dependent breast cancer research *in vitro*. This step alters the MCF-7 cells biomarker expression and thus subsequent treatments performed on these cells can have effects contrasting to those in which this step was overlooked.

Hormone-therapy treatments produced an increased cytoplasmic ER α expression, which is associated with hormone-therapy resistance. However, this was partially ameliorated with the addition of the simultaneous hormone treatment, indicating an oestrogen and progesterone hormonal influence in ER α expression. Hormone-therapy produces variable effects on MUC1 expression, with Anastrozole increasing and RU486 decreasing MUC1 expression. This, again, was partially ameliorated under hormonal mediation, indicating that MUC1 expression also is influenced with oestrogen and progesterone. Hormone therapy treatments caused a decrease in PR expression, which was further reduced with the Anastrozole treatment under hormone mediation. Interestingly, under hormone mediation, RU486 induces an increase in PR expression, indicating an interaction between these three compounds. Notably, the combination of Anastrozole hormone-therapy in a continued simulated hormone environment results in a decrease in all three biomarkers, this is a phenotypic change associated with metastatic processes, which is contra-indicated with regards to breast cancer treatment.

The addition of immunotherapy to the hormone-therapy treatment produced a decrease of all three biomarkers. Conversely, NK cell mediation under hormonal influence, results in an increase in ER α , MUC1 and nuclear PR expression with a decrease in cytoplasmic PR expression. These results indicate that NK cells mediate the expression of these biomarkers depending on the hormonal milieu. This also indicates that NK cell influence may cause MCF-7 cells to mediate PR expression between cytoplasmic and nuclear compartments.

Increased PR expression or MUC1 homogenous expression is associated with genomic effects including oncogene transcription as well as inhibition of apoptosis. Furthermore these biomarkers also interact with ER α and are thus also implicated in ER α mediated tumour progression. MUC1 has also been associated with immune evasion which is essential for metastasis. Therefore, not only is the level of biomarker expression important, but so is the cellular location in breast cancer diagnosis and treatment. There also seems to be a more definite role for progesterone in hormone-therapy as well as NK cell immunotherapy than currently apparent. The effect of hormones and hormone-therapy on NK cells and other cells of the immune system should also be taken into account when treating hormone-dependent breast cancer. These results may explain why despite advances in treatment strategies, overall survival remains poor with recurrence common. An expanded study is needed looking at longer hormone-therapy treatment times to mimic the clinical conditions in which these drugs are used, and to validate the postulations of this study.

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APPENDIX A: ETHICS CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

R14/49 Augustine

CLEARANCE CERTIFICATE

PROTOCOL NUMBER M081036

PROJECT

Characterisation and Analysis of the
in vitro Anti-Tumorigenic Capacity of
Tumour Infiltrating Lymphocytes and
Peripheral Blood Lymphocytes Obtained
from Breast Cancer Patients

INVESTIGATORS

Ms TN Augustine

DEPARTMENT

School of Anatomical Sciences

DATE CONSIDERED

08.10.31

DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.01.30

CHAIRPERSON



(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr G Candy

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

University
of the Witwatersrand,
Johannesburg



01 June 2011

Ms Tanya Augustine
Lecturer
School of anatomical Sciences
Medical School
University

Dear Ms Augustine

**RE: Protocol M08103: 'Characterisation and Analysis of
Lymphocytes in Patients with Breast Diseases
Protocol Amendment**

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has reviewed and approved the following amendments on the abovementioned protocol as detailed in your letter dated 25 March 2011:

- Obtaining patients samples from CM Johannesburg Academic Hospital, Oncology Clinic (with written permission)
- Verification of data obtained
- Inclusion of Ms J Gill as sub-investigator

Thank you for keeping us informed and updated.

Yours sincerely,

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

APPENDIX B: RECIPES

B1. Media A

- 45ml DMEM (Lonza 12-604)
- 5ml FBS
- 50µl P/S

All were added together in 50 ml tubes under sterile conditions and stored at 4°C.

B2. Cryopreservation media

- 10% DMSO
- 60% FBS
- 30% DMEM (Lonza 12-604)

Mixed all together in a 10 ml centrifuge tube and stored at -20°C.

B3. Phosphate Buffered Solution (PBS)

- 8g sodium chloride (NaCl)
- 0.2g potassium chloride (KCl)
- 1.44g di-sodium hydrogen orthophosphate (Na_2HPO_4)
- 0.24g potassium di-hydrogen orthophosphate (KH_2PO_4)
- 1l dH₂O

All the solids were dissolved in 800 ml dH₂O and the pH adjusted to 7.4.

This was then adjusted to 1l with additional dH₂O and autoclaved. Stored at 4°C.

B4. Media B

- 45ml Phenol red free DMEM (Lonza 12-917)
- 5ml DCC-stripped FBS
- 50µl P/S

All were added together in 50 ml tubes under sterile conditions and stored at 4°C.

B5. DCC-stripped FBS

- 20ml Tris-HCl (0.1 M, pH 8.0)
- 180ml dH₂O
- 0.05g dextran T500
- 0.5g activated charcoal powder
- 80ml Heat inactivated FBS

Added all together except FBS and mixed overnight at 4°C in an airtight container.

Filled 8 10ml tubes with suspension and centrifuged at 1000x g for 20 min.

Discarded supernatant, refilled tubes with DCC suspension and repeated centrifugation.

Discarded supernatant and added 10 ml FBS to each tube.

Repeated centrifugation, sterile filtered supernatant with 0.2 µm filter and discarded pellet.

Stored at -20°C.

B6. 0.1 M Tris-HCl

- 1.22g Tris
- 100ml dH₂O
- 2M HCl

Dissolved Tris in 80 ml dH₂O and adjusted the pH to 8.0 with the HCl.

Adjusted this to 100 ml with additional dH₂O.

B7. 1000x treatment stock solutions (1mM)

- 0.0029g Anastrozole powder
- 0.0043g RU486 powder
- 0.0027g 17β-oestradiol powder
- 0.0031g progesterone powder
- 1ml DMSO (x4)
- 9ml Media B (x4)

To each labelled 10 ml centrifuge tubes (see below), 1 ml DMSO was added.

To each tube, the appropriate powders (hormone/drugs/none) were added and dissolved (see below).

To each tube 9 ml Media B was added.

Each solution was sterilised with a 0.2 µm filter and stored at -20°C.

ANAS = 1 ml DMSO + 9 ml Media B + anastrozole powder

RU486 = 1 ml DMSO + 9 ml Media B + RU486 powder

EP = 1 ml DMSO + 9 ml Media B + 17β-oestradiol powder + progesterone powder

dDMSO = 1 ml DMSO + 9 ml Media B

B8. Miltenyi buffer

- 100ml PBS
- 0.5g BSA
- 2mM EDTA

The BSA and EDTA were dissolved in the PBS.

Stored at 4°C.

B9. Activation Media A

- RPMI 1640
- 10% FBS
- 0.1% P/S
- 1 µg/ml phytohaemagglutinin (PHA)
- 0.8 ng/ml Interleukin-2 (IL-2)

All were added together and used immediately.

B10. Activation Media B

- RPMI 1640
- 10% FBS
- 0.1% P/S
- 0.8 ng/ml IL-2

B11. Antigen Retrieval Buffer (ARB)

- 100ml dH₂O
- 1.22g Tris(hydroxymethyl)aminomethane
- 5g urea

The Tris and urea were dissolved in 80 ml dH₂O.

The pH was adjusted to 9.5.

This was adjusted to 100 ml and stored at 4°C.

APPENDIX C: SUPPLEMENTARY IMAGES

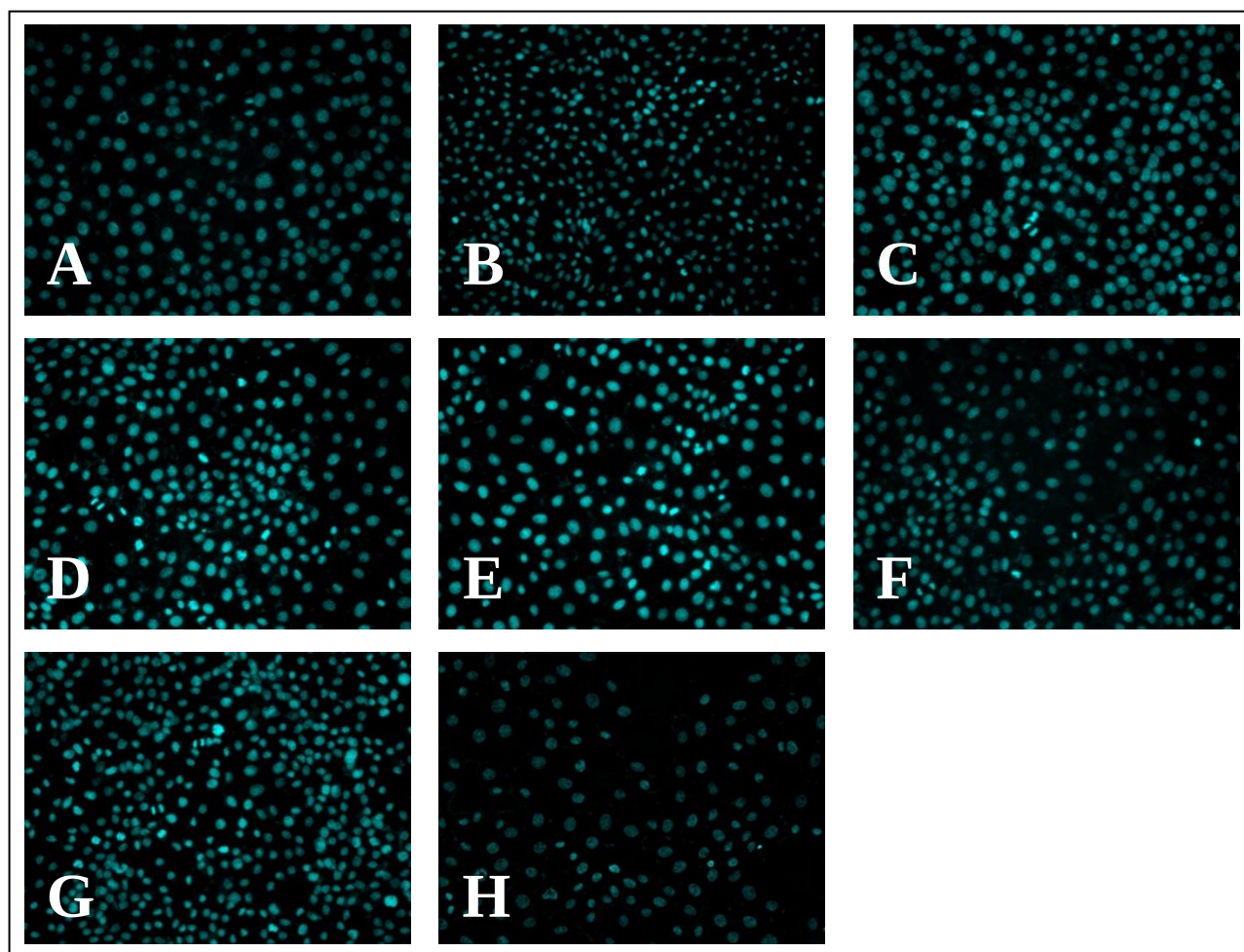


Figure C1. Representative photomicrographs showing the ICC controls

Treatment groups: **A:** negative control **B:** MUC1 primary antibody control **C:** ER α primary antibody control **D:** PR primary antibody control **E:** Alexa Fluor® 488 secondary antibody control **F:** Alexa Fluor® 594 secondary antibody control **G:** IgG isotype control **H:** IgG3 isotype control. That no fluorescence is noted bar the DAPI stain confirms the efficacy of the technique. Fluorescent microscopy, 40x magnification.

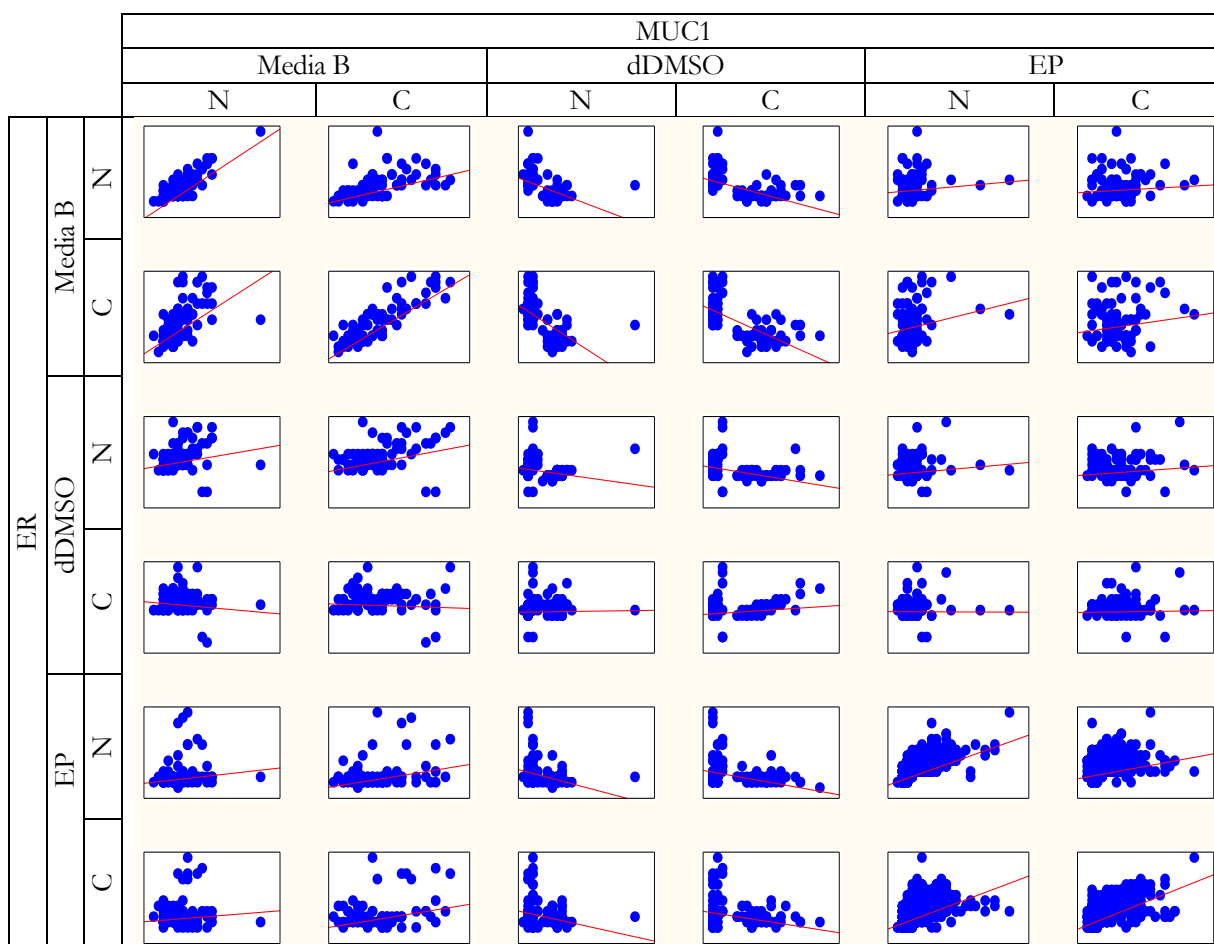


Figure C2. The graphs for MUC1 and ER α correlation in the nucleus (N) and cytoplasm (C) under treatment with the controls

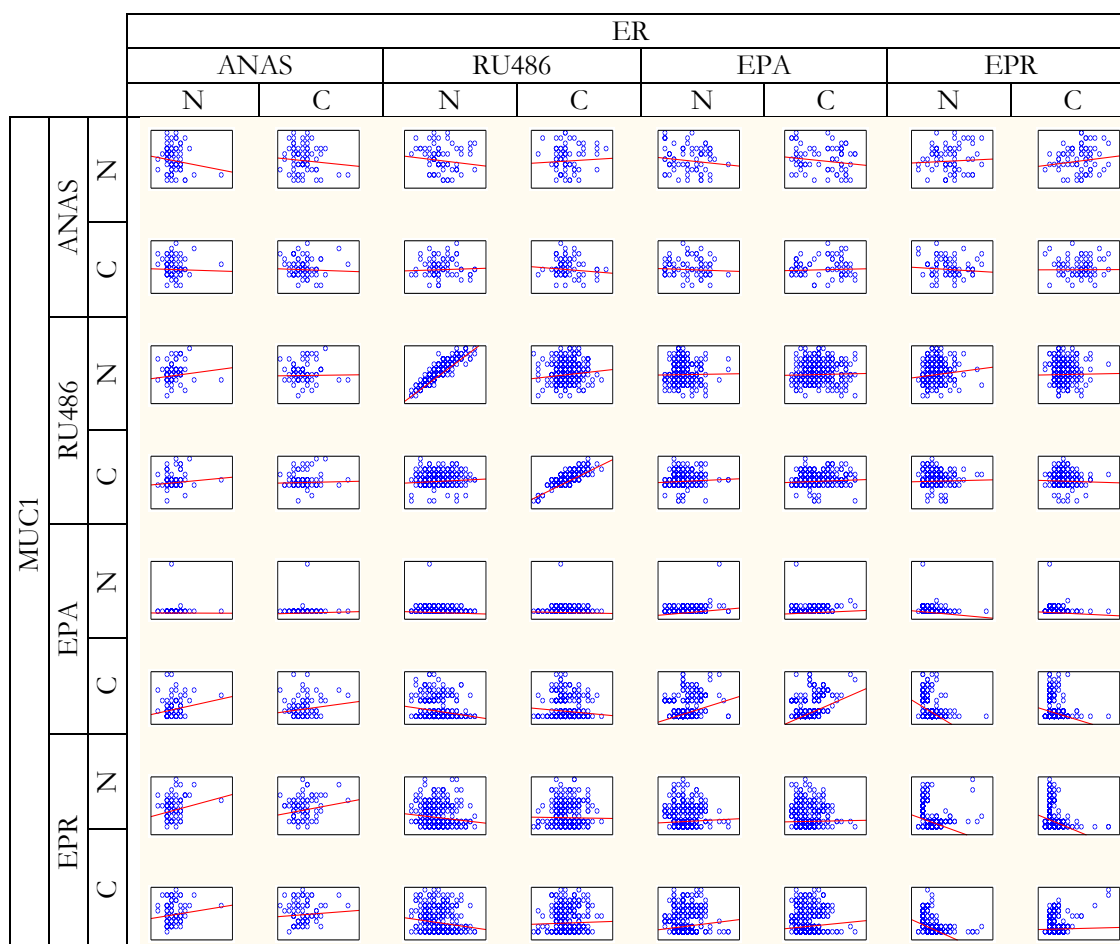


Figure C3. The graphs for MUC1 and ER α correlation in the nucleus (N) and cytoplasm (C) under treatment with hormone-therapy

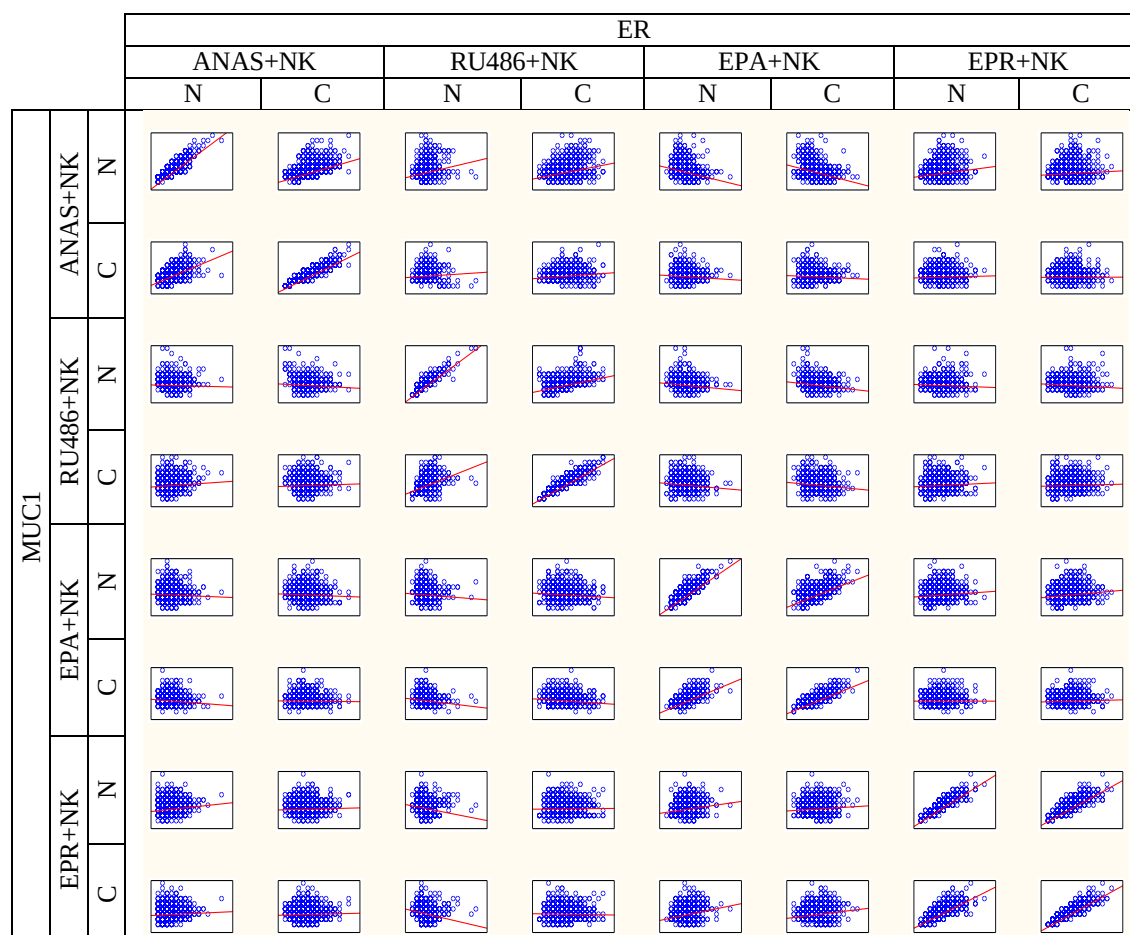


Figure C4. The graphs for MUC1 and ER α correlation in the nucleus (N) and cytoplasm (C) under treatment with combined hormone-therapy and immunotherapy

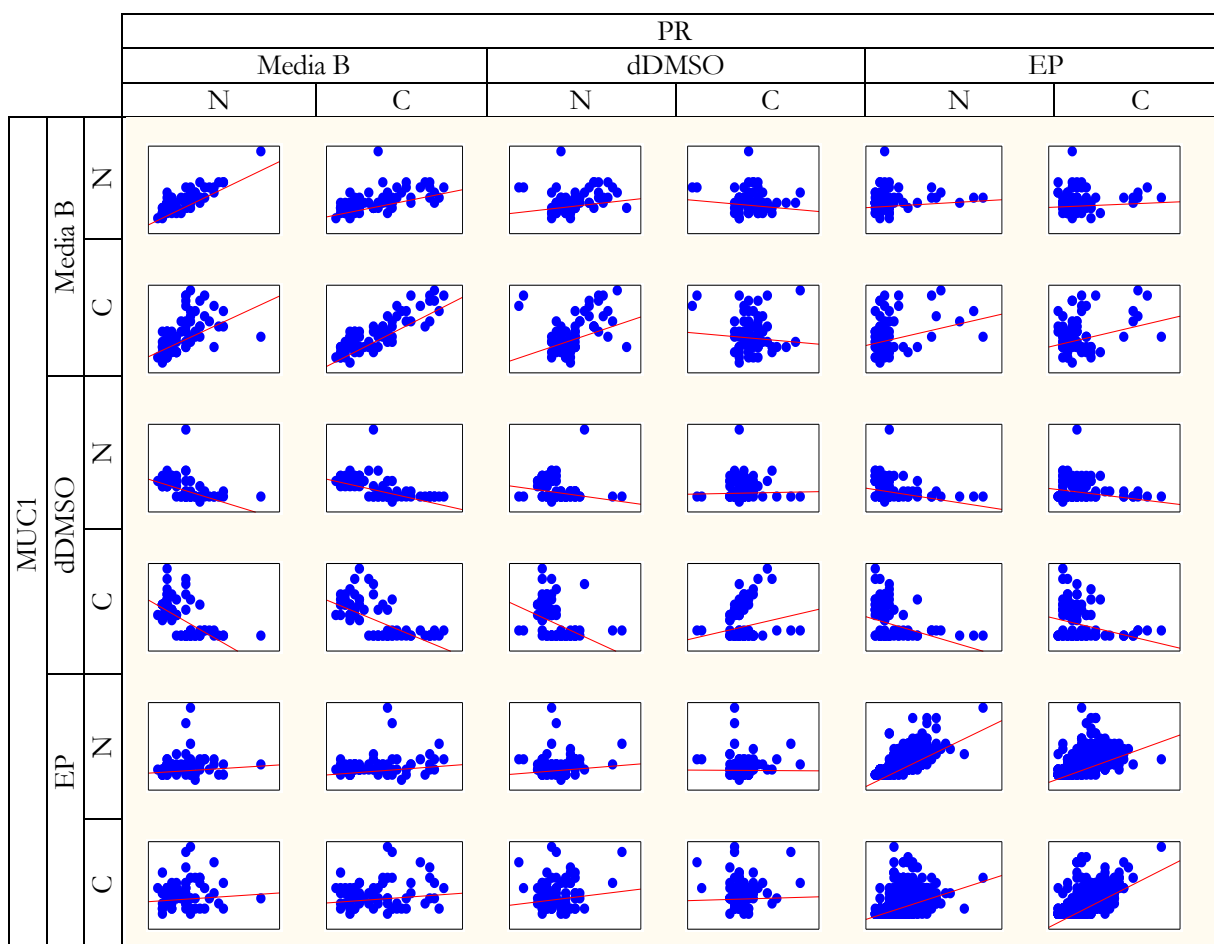


Figure C5. The graphs for MUC1 and PR correlation in the nucleus (N) and cytoplasm (C) under treatment with controls

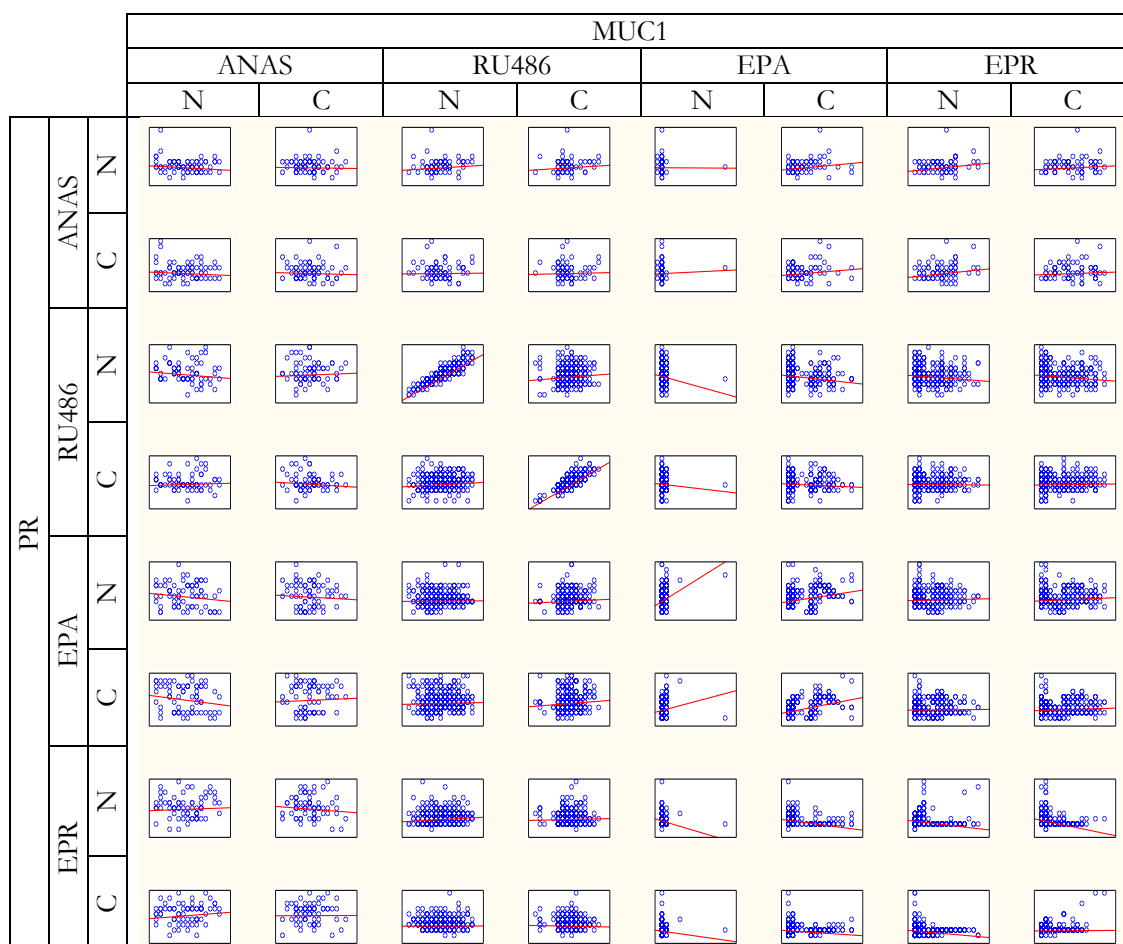


Figure C6. The graphs for MUC1 and PR correlation in the nucleus (N) and cytoplasm (C) under hormone-therapy treatment

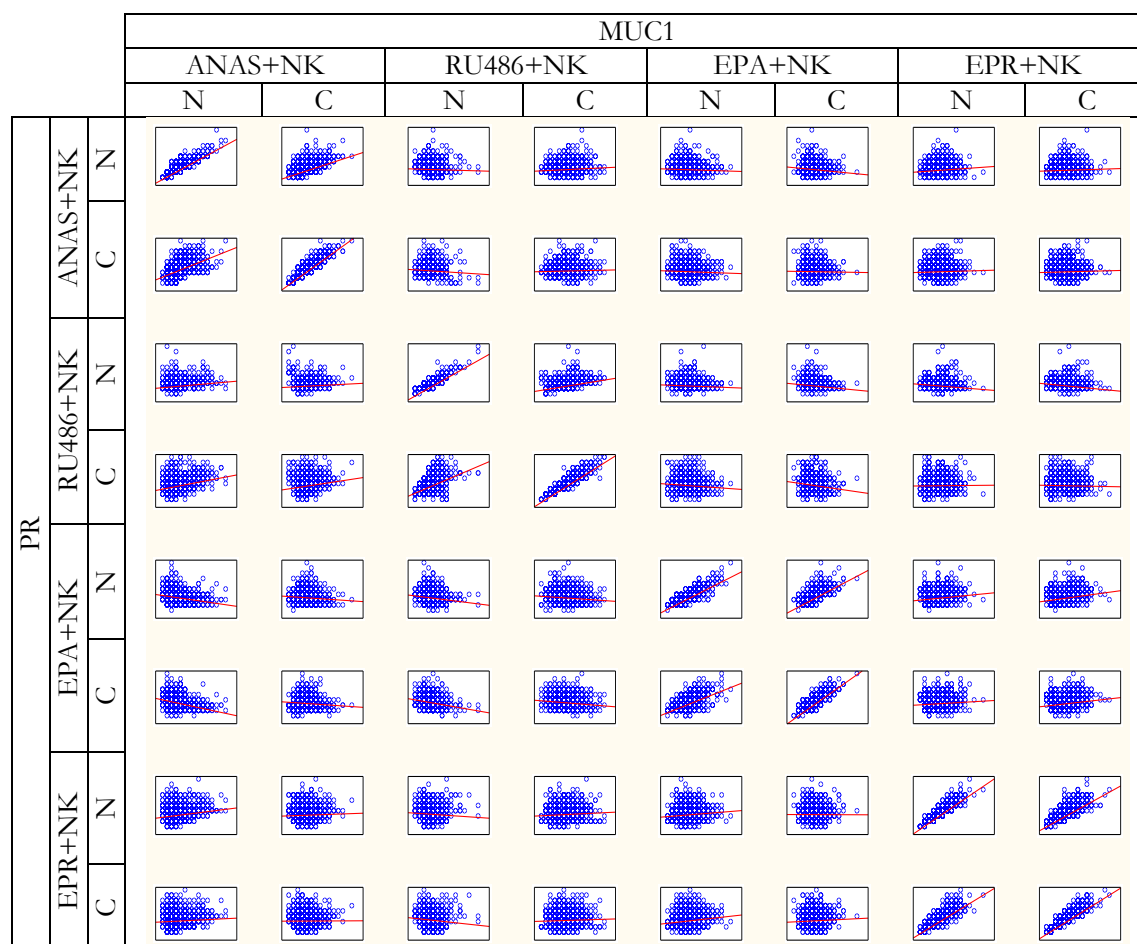


Figure C7. The graphs for MUC1 and PR correlation in the nucleus (N) and cytoplasm (C) under combined hormone-therapy and immunotherapy treatments

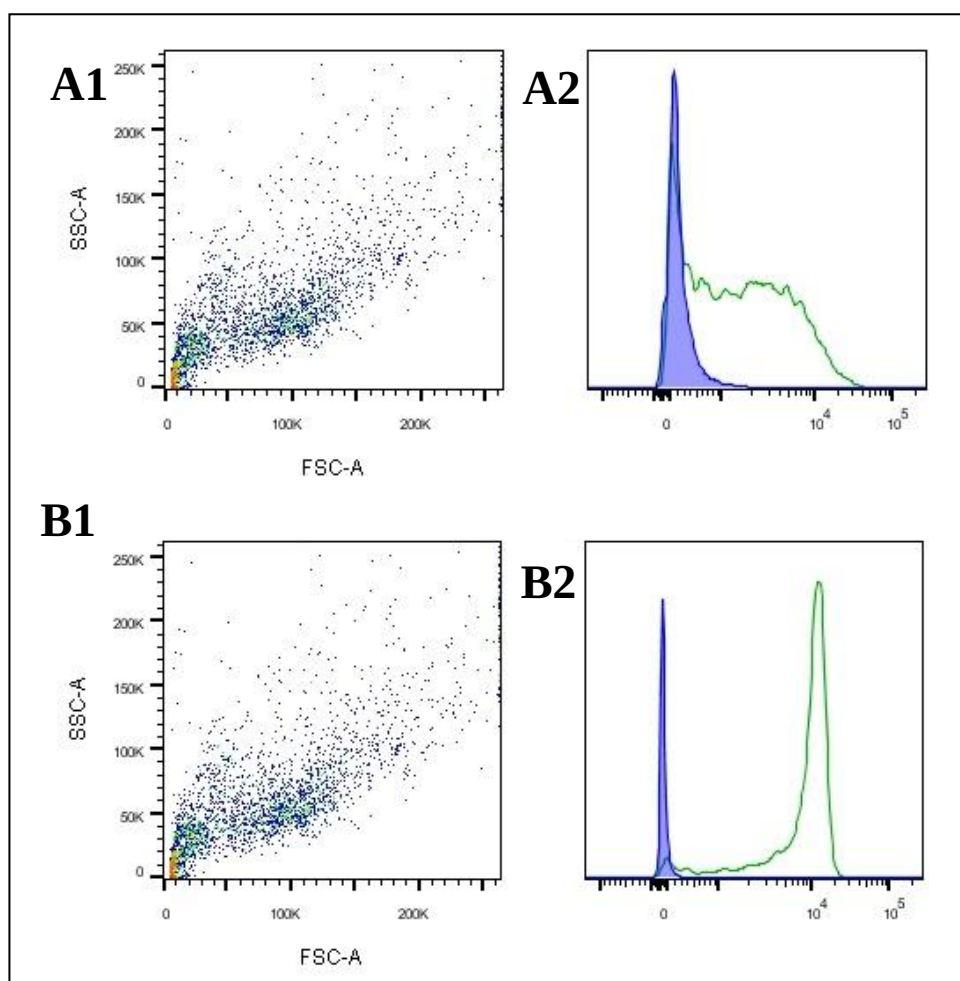


Figure C8. Compensation controls for the Annexin V-PI flow cytometric analysis

A: Annexin V-FITC stained cells **B:** PI stained cells **1:** Scatterplots of SSC versus FSC **2:** Histograms with single stained samples (green) unstained samples (blue).

APPENDIX D: DATA TABLES

Table D1. The PR expression MFI values

Treatment	Overall (Cell)	Cytoplasm	Nucleus
dDMSO	0.066359081	0.063169406	0.087405914
EP	0.015219127	0.014652951	0.01941196
ANAS	0.013463791	0.013228454	0.014575683
RU486	0.016541366	0.016043812	0.019008076
EPA	0.010852946	0.010710104	0.011970915
EPR	0.099298576	0.081116268	0.252515427
dDMSO+NK	0.009459212	0.009426827	0.009787098
EP+NK	0.009307124	0.009224041	0.009583287
ANAS+NK	0.009133525	0.009427954	0.009426509
RU486+NK	0.008764956	0.009184811	0.009093418
EPA+NK	0.009525461	0.009066107	0.009733158
EPR+NK	0.009243808	0.008731791	0.009467947

Table D2. The p-values for the normality tests performed on the PR data ($p < 0.05$)

Treatment	Category	Shapiro-Wilk	Jaque-Bera	Chi ²	Anderson-Darling
dDMSO	Overall	0.26	0.3841	0.43858	0.3568
	Cytoplasm	0.2364	0.3499	0.62101	0.2592
	Nucleus	0.04134	0.05167	0.22239	0.2459
EP	Overall	0.96	0.9093	0.69489	0.9293
	Cytoplasm	0.9314	0.9188	0.38046	0.8723
	Nucleus	0.07439	0.02842	0.087321	0.1143
ANAS	Overall	0.801	0.341	0.273	0.1611
	Cytoplasm	0.08768	0.2541	0.41422	0.147
	Nucleus	0.2506	0.4619	0.52709	0.2938
RU486	Overall	0.0605	0.2886	0.37978	0.0991
	Cytoplasm	0.03949	0.2453	0.22739	0.0552
	Nucleus	0.5307	0.6582	0.7697	0.6098
EPA	Overall	0.2867	0.457	0.33075	0.5454
	Cytoplasm	0.3654	0.4752	0.17841	0.6509
	Nucleus	0.2546	0.2695	0.58558	0.2714
EPR	Overall	0.01249	0.03536	0.002326	0.01467
	Cytoplasm	0.454	0.5447	0.46018	0.5934
	Nucleus	0.000677	0.1056	0.078731	0.000312
dDMSO+NK	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	<0.0001	<0.001	<0.001	<0.005
EP+NK	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	<0.0001	<0.001	<0.001	<0.005
ANAS+NK	Overall	1.17E-21	0	3.41E-09	2.81E-24
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	1.26E-17	0	2.50E-05	3.06E-22
RU486+NK	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	6.42E-25	0	4.29E-12	1.49E-59
EPA+NK	Overall	2.23E-27	0	1.05E-11	2.27E-48
	Cytoplasm	1.70E-27	0	5.74E-13	3.70E-54
	Nucleus	1.23E-25	0	1.46E-08	3.70E-39
EPR+NK	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	3.28E-11	0	8.10E-06	0.003255
	Nucleus	1.22E-08	0	4.66E-05	0.000607

Table D3. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy PR data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

		ANAS			DMSO			EP			EPA			EPR			RU486		
		O	N	C	O	N	C	O	N	C	O	N	C	O	N	C	O	N	C
ANAS	O		0.0009521	0.3042	3.00E-13	3.00E-13	3.00E-13	2.84E-07	1.56E-10	0.0002568	4.85E-11	5.39E-07	4.09E-11	1.04E-09	1.04E-09	1.04E-09	2.05E-09	3.78E-11	2.91E-08
	N	0.4142		8.66E-05	3.00E-13	3.00E-13	3.00E-13	0.06223	1.74E-10	0.9672	9.42E-12	4.89E-10	7.24E-12	1.04E-09	1.04E-09	1.04E-09	7.68E-05	1.00E-08	0.002122
	C	1	0.03769		3.00E-13	3.00E-13	3.00E-13	4.28E-08	1.56E-10	6.49E-06	1.84E-10	3.38E-06	1.03E-10	1.04E-09	1.04E-09	1.04E-09	1.54E-10	1.85E-11	1.89E-09
DMSO	O	1.30E-10	1.30E-10	1.30E-10		1.58E-06	0.2518	3.03E-12	3.03E-12	3.03E-12	8.98E-15	8.98E-15	8.98E-15	5.13E-10	4.05E-11	7.97E-06	2.30E-14	2.30E-14	2.30E-14
	N	1.30E-10	1.30E-10	1.30E-10	0.0006851		3.24E-08	3.03E-12	3.03E-12	3.03E-12	8.98E-15	8.98E-15	8.98E-15	0.0007856	4.05E-11	0.2168	2.30E-14	2.30E-14	2.30E-14
	C	1.30E-10	1.30E-10	1.30E-10	1	1.41E-05		3.03E-12	3.03E-12	3.03E-12	8.98E-15	8.98E-15	8.98E-15	7.58E-11	4.05E-11	4.34E-07	2.30E-14	2.30E-14	2.30E-14
EP	O	0.0001234	1	1.86E-05	1.32E-09	1.32E-09	1.32E-09		1.16E-09	0.04507	1.97E-11	2.88E-11	1.97E-11	3.47E-09	3.47E-09	3.47E-09	0.0114	6.35E-07	0.1427
	N	6.80E-08	7.57E-08	6.80E-08	1.32E-09	1.32E-09	1.32E-09	5.06E-07		7.35E-10	1.97E-11	1.97E-11	1.97E-11	3.47E-09	3.47E-09	3.47E-09	3.17E-06	0.5354	1.58E-07
	C	0.1117	1	0.002824	1.32E-09	1.32E-09	1.32E-09	1	3.20E-07		1.97E-11	9.74E-11	1.97E-11	3.47E-09	3.47E-09	3.47E-09	0.0001079	1.73E-08	0.003145
EPA	O	2.11E-08	4.10E-09	8.02E-08	3.91E-12	3.91E-12	3.91E-12	8.57E-09	8.57E-09	8.57E-09		5.90E-07	0.43	1.87E-10	1.87E-10	1.87E-10	3.11E-13	3.11E-13	3.11E-13
	N	0.0002343	2.13E-07	0.00147	3.91E-12	3.91E-12	3.91E-12	1.26E-08	8.57E-09	4.24E-08	0.0002565		4.23E-08	1.87E-10	1.87E-10	1.87E-10	3.68E-13	3.38E-13	4.72E-13
	C	1.78E-08	3.15E-09	4.49E-08	3.91E-12	3.91E-12	3.91E-12	8.57E-09	8.57E-09	8.57E-09	1	1.84E-05		1.87E-10	1.87E-10	1.87E-10	3.11E-13	3.11E-13	3.11E-13
EPR	O	4.53E-07	4.53E-07	4.53E-07	2.23E-07	0.3417	3.30E-08	1.51E-06	1.51E-06	1.51E-06	8.13E-08	8.13E-08	8.13E-08		1.44E-08	1.44E-08	2.93E-10	2.93E-10	2.93E-10
	N	4.53E-07	4.53E-07	4.53E-07	1.76E-08	1.76E-08	1.76E-08	1.51E-06	1.51E-06	1.51E-06	8.13E-08	8.13E-08	8.13E-08	6.26E-06		1.44E-08	2.93E-10	2.93E-10	2.93E-10
	C	4.53E-07	4.53E-07	4.53E-07	0.003467	1	0.0001888	1.51E-06	1.51E-06	1.51E-06	8.13E-08	8.13E-08	8.13E-08	6.26E-06	6.26E-06		2.93E-10	2.93E-10	2.93E-10
RU486	O	8.91E-07	0.03339	6.68E-08	9.99E-12	9.99E-12	9.99E-12	1	0.001378	0.04695	1.35E-10	1.60E-10	1.35E-10	1.27E-07	1.27E-07	1.27E-07		0.0003402	0.2645
	N	1.65E-08	4.35E-06	8.03E-09	9.99E-12	9.99E-12	9.99E-12	0.000276	1	7.54E-06	1.35E-10	1.47E-10	1.35E-10	1.27E-07	1.27E-07	1.27E-07	0.148		2.48E-05
	C	1.27E-05	0.923	8.22E-07	9.99E-12	9.99E-12	9.99E-12	1	6.88E-05	1	1.35E-10	2.05E-10	1.35E-10	1.27E-07	1.27E-07	1.27E-07	1	0.01078	

Table D4. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy and immunotherapy PR data ($p < 0.001$) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

		ANAS+NK			DMSO+NK			EP+NK			EPA+NK			EPR+NK			RU486+NK		
		O	N	C	O	N	C	O	N	C	O	N	C	O	N	C	O	N	C
ANAS+NK	O		9.16E-127	1.30E-21	4.94E-70	2.27E-206	2.82E-55	3.06E-94	1.99E-224	1.45E-41	3.68E-185	1.734E-310	6.65E-132	7.00E-32	2.11E-146	3.84E-11	1.07E-221	8.47E-16	2.15E-246
	N	3.99E-124		3.83E-182	0.08086	4.27E-56	0.0002801	1.28E-25	4.33E-23	2.33E-76	5.84E-09	1.22E-104	0.369	1.38E-47	0.02882	1.74E-84	1.13E-277	1.34E-82	7.86E-285
	C	5.67E-19	1.67E-179		2.33E-115	3.13E-233	1.28E-98	8.46E-172	5.40E-275	9.11E-117	3.61E-246	0	1.30E-198	4.82E-78	5.37E-207	1.02E-44	7.54E-188	1.02E-06	4.28E-221
DMSO+NK	O	2.15E-67	1	1.01E-112		7.60E-43	0.09662	1.74E-07	3.46E-20	6.19E-30	2.03E-09	4.74E-71	0.3128	2.48E-23	0.003287	5.99E-44	3.98E-230	1.69E-65	3.08E-240
	N	9.86E-204	1.86E-53	1.36E-230	3.31E-40		1.13E-50	3.73E-138	2.12E-18	4.17E-194	1.47E-32	0.01497	1.63E-65	1.10E-159	3.36E-49	5.56E-197	3.47E-257	3.33E-139	8.17E-259
	C	1.23E-52	0.1218	5.58E-96	1	4.90E-48		0.004125	8.69E-28	6.24E-19	5.19E-15	9.54E-82	0.00277	3.22E-15	1.71E-06	2.37E-32	3.08E-224	1.46E-58	5.59E-236
EP+NK	O	1.33E-91	5.58E-23	3.68E-169	7.55E-05	1.62E-135	1		1.40E-109	2.49E-28	9.53E-69	9.36E-248	5.38E-23	8.50E-10	2.79E-40	2.28E-36	4.724E-317	1.64E-60	0
	N	8.66E-222	1.89E-20	2.35E-272	1.51E-17	9.23E-16	3.78E-25	6.08E-107		4.64E-194	2.10E-05	8.35E-46	1.16E-30	3.89E-144	2.66E-16	1.71E-201	0	3.23E-140	0
	C	6.31E-39	1.02E-73	3.97E-114	2.69E-27	1.82E-191	2.71E-16	1.08E-25	2.02E-191		1.47E-140	0	8.76E-76	0.008753	7.50E-99	0.0001791	6.52E-305	7.76E-39	1.419E-319
EPA+NK	O	1.60E-182	2.54E-06	1.57E-243	8.83E-07	6.38E-30	2.26E-12	4.15E-66	0.009138	6.40E-138		5.74E-74	3.31E-13	2.54E-107	5.20E-05	1.87E-161	0	8.21E-129	0
	N	7.54E-308	5.31E-102	0	2.06E-68	1	4.15E-79	4.07E-245	3.63E-43	0	2.50E-71		2.17E-132	1.36E-295	1.56E-106	0	0	1.69E-215	0
	C	2.89E-129	1	5.67E-196	1	7.08E-63	1	2.34E-20	5.05E-28	3.81E-73	1.44E-10	9.44E-130		2.71E-52	0.001595	1.53E-95	0	6.48E-99	0
EPR+NK	O	3.05E-29	6.00E-45	2.10E-75	1.08E-20	4.77E-157	1.40E-12	3.70E-07	1.69E-141	1	1.11E-104	5.90E-293	1.18E-49		5.38E-74	1.14E-09	2.19E-288	5.23E-43	1.909E-312
	N	9.17E-144	1	2.34E-204	1	1.46E-46	0.0007435	1.21E-37	1.16E-13	3.26E-96	0.02262	6.76E-104	0.6937	2.34E-71		4.22E-121	0	7.24E-111	0
	C	1.67E-08	7.58E-82	4.44E-42	2.60E-41	2.42E-194	1.03E-29	9.92E-34	7.46E-199	0.07789	8.14E-159	0	6.64E-93	4.94E-07	1.84E-118		3.09E-264	1.30E-27	2.16E-292
RU486+NK	O	4.66E-219	4.89E-275	3.28E-185	1.73E-227	1.51E-254	1.34E-221	2.055E-314	0	2.84E-302	0	0	0	9.54E-286	0	1.34E-261		6.75E-77	0.0001995
	N	3.68E-13	5.82E-80	0.0004417	7.37E-63	1.45E-136	6.33E-56	7.11E-58	1.41E-137	3.38E-36	3.57E-126	7.34E-213	2.82E-96	2.28E-40	3.15E-108	5.64E-25	2.94E-74		7.33E-98
	C	9.35E-244	3.42E-282	1.86E-218	1.34E-237	3.55E-256	2.43E-233	0	0	6.172E-317	0	0	0	8.303E-310	0	9.38E-290	0.08676	3.19E-95	

Table D5. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy compared to the hormone-therapy and immunotherapy PR expression nucleus data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

	ANAS+NK	DMSO+NK	EP+NK	EPA+NK	EPR+NK	RU486+NK	ANAS	DMSO	EP	EPA	EPR	RU486
ANAS+NK		4.27E-56	4.33E-23	1.22E-104	0.02882	1.34E-82	1.02E-20	7.88E-30	3.11E-18	5.52E-25	1.01E-15	8.64E-24
DMSO+NK	2.82E-54		2.12E-18	0.01497	3.36E-49	3.33E-139	2.13E-20	1.92E-29	5.27E-18	4.49E-24	1.27E-15	1.99E-23
EP+NK	2.86E-21	1.40E-16		8.35E-46	2.66E-16	3.23E-140	8.85E-21	3.18E-30	2.27E-18	6.29E-25	7.97E-16	5.30E-24
EPA+NK	8.06E-103	0.9879	5.51E-44		1.56E-106	1.69E-215	5.91E-21	2.18E-30	1.98E-18	6.34E-25	7.24E-16	3.89E-24
EPR+NK	1	2.22E-47	1.75E-14	1.03E-104		7.24E-111	5.91E-21	1.89E-30	1.89E-18	3.09E-25	6.98E-16	3.67E-24
RU486+NK	8.83E-81	2.20E-137	2.13E-138	1.11E-213	4.78E-109		1.09E-20	9.01E-30	3.26E-18	6.73E-25	1.04E-15	9.39E-24
ANAS	6.73E-19	1.41E-18	5.84E-19	3.90E-19	3.90E-19	7.16E-19		3.00E-13	1.74E-10	4.89E-10	1.04E-09	1.00E-08
DMSO	5.20E-28	1.27E-27	2.10E-28	1.44E-28	1.25E-28	5.95E-28	1.98E-11		3.03E-12	8.98E-15	4.05E-11	2.30E-14
EP	2.05E-16	3.48E-16	1.50E-16	1.31E-16	1.25E-16	2.15E-16	1.15E-08	2.00E-10		1.97E-11	3.47E-09	0.5354
EPA	3.64E-23	2.97E-22	4.15E-23	4.19E-23	2.04E-23	4.44E-23	3.23E-08	5.93E-13	1.30E-09		1.87E-10	3.38E-13
EPR	6.64E-14	8.37E-14	5.26E-14	4.78E-14	4.60E-14	6.87E-14	6.88E-08	2.68E-09	2.29E-07	1.23E-08		2.93E-10
RU486	5.70E-22	1.31E-21	3.50E-22	2.57E-22	2.42E-22	6.20E-22	6.60E-07	1.52E-12	1	2.23E-11	1.93E-08	

Table D6. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy compared to the combined hormone-therapy and immunotherapy PR expression cytoplasm data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

	ANAS+NK	DMSO+NK	EP+NK	EPA+NK	EPR+NK	RU486+NK	ANAS	DMSO	EP	EPA	EPR	RU486
ANAS+NK		1.28E-98	9.11E-117	1.30E-198	1.02E-44	4.28E-221	1.02E-20	7.88E-30	3.11E-18	1.07E-24	1.01E-15	8.64E-24
DMSO+NK	8.46E-97		6.24E-19	0.00277	2.37E-32	5.59E-236	1.90E-20	1.92E-29	4.34E-18	1.58E-18	1.27E-15	1.52E-23
EP+NK	6.02E-115	4.12E-17		8.76E-76	0.0001791	1.419E-319	7.06E-21	3.18E-30	2.26E-18	5.03E-24	7.97E-16	4.92E-24
EPA+NK	8.60E-197	0.1828	5.78E-74		1.53E-95	0	5.95E-21	2.18E-30	1.98E-18	1.32E-19	7.24E-16	3.89E-24
EPR+NK	6.74E-43	1.57E-30	0.01182	1.01E-93		2.16E-292	5.27E-21	1.89E-30	1.88E-18	1.05E-23	6.98E-16	3.56E-24
RU486+NK	2.82E-219	3.69E-234	9.364E-318	0	1.42E-290		1.09E-20	9.01E-30	3.26E-18	7.04E-25	1.04E-15	9.39E-24
ANAS	6.73E-19	1.26E-18	4.66E-19	3.93E-19	3.48E-19	7.16E-19		3.00E-13	6.49E-06	1.03E-10	1.04E-09	1.89E-09
DMSO	5.20E-28	1.27E-27	2.10E-28	1.44E-28	1.25E-28	5.95E-28	1.98E-11		3.03E-12	8.98E-15	4.34E-07	2.30E-14
EP	2.05E-16	2.87E-16	1.49E-16	1.31E-16	1.24E-16	2.15E-16	0.0004285	2.00E-10		1.97E-11	3.47E-09	0.003145
EPA	7.08E-23	1.04E-16	3.32E-22	8.72E-18	6.92E-22	4.64E-23	6.82E-09	5.93E-13	1.30E-09		1.87E-10	3.11E-13
EPR	6.64E-14	8.37E-14	5.26E-14	4.78E-14	4.60E-14	6.87E-14	6.88E-08	2.86E-05	2.29E-07	1.23E-08		2.93E-10
RU486	5.70E-22	1.00E-21	3.25E-22	2.57E-22	2.35E-22	6.20E-22	1.25E-07	1.52E-12	0.2075	2.05E-11	1.93E-08	

Table D7. The MFI values for the ER α expression

Treatment	Overall (Cell)	Cytoplasm	Nucleus
Media B	0.05485177	0.05016156	0.07280618
dDMSO	0.009204749	0.009096929	0.009720602
EP	0.008494155	0.008478679	0.008594465
ANAS	0.054962757	0.0512726	0.07391729
RU486	0.009998437	0.010004132	0.010010256
EPA	0.008764179	0.008721687	0.009100168
EPR	0.008891058	0.008843711	0.009193471
dDMSO+NK	0.008408984	0.008327775	0.008717904
EP+NK	0.009195583	0.009118333	0.009411306
ANAS+NK	0.009053005	0.008991113	0.009225404
RU486+NK	0.008992182	0.008917346	0.009236711
EPA+NK	0.009152391	0.009103248	0.009317897
EPR+NK	0.009275839	0.009203269	0.0094431

Table D8. The p-values for the normality tests performed on the ER α data

Treatment	Category	Shapiro-Wilk	Jaque-Bera	Chi ²	Anderson-Darling
dDMSO	Overall	1.62E-09	7.53E-69	4.78E-07	5.75E-11
	Cytoplasm	4.72E-08	4.12E-28	0.00016559	1.03E-09
	Nucleus	7.24E-06	1.45E-16	3.37E-02	8.04E-05
EP	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	<0.0001	<0.001	<0.001	<0.005
ANAS	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	<0.0001	<0.001	<0.001	<0.005
RU486	Overall	1.41E-02	1.20E-01	0.2673	1.09E-02
	Cytoplasm	0.001892	0.0169	0.11195	0.001038
	Nucleus	0.009197	0.0148	0.10188	0.02716
EPA	Overall	1.58E-13	4.35E-58	6.06E-07	5.72E-16
	Cytoplasm	1.42E-12	5.64E-56	0.00018435	1.60E-11
	Nucleus	2.79E-16	4.12E-126	5.68E-12	2.50E-24
EPR	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	<0.0001	<0.001	<0.001	<0.005
dDMSO+NK	Overall	7.10E-47	0	0	2.19E-135
	Cytoplasm	3.28E-47	0	0	2.72E-125
	Nucleus	3.14E-45	0	0	4.45E-170
EP+NK	Overall	5.50E-23	8.97E-224	5.32E-06	1.32E-27
	Cytoplasm	5.97E-25	0	1.75E-05	1.13E-31
	Nucleus	<0.0001	<0.001	<0.001	<0.005
ANAS+NK	Overall	6.36E-15	5.98E-30	0.00053098	2.15E-18
	Cytoplasm	1.27E-12	2.80E-19	6.20E-06	1.52E-14
	Nucleus	8.41E-28	1.49E-296	1.97E-10	4.22E-50
RU486+NK	Overall	1.20E-10	7.03E-12	0.0032746	7.63E-12
	Cytoplasm	1.53E-12	1.07E-16	0.0016766	1.24E-15
	Nucleus	3.49E-26	0	3.63E-07	2.08E-39
EPA+NK	Overall	6.43E-13	1.19E-09	8.74E-10	8.31E-21
	Cytoplasm	1.48E-13	1.97E-09	1.67E-12	1.63E-23
	Nucleus	1.09E-12	8.74E-25	0.019309	1.18E-09
EPR+NK	Overall	8.28E-13	5.52E-18	0.001705	3.12E-10
	Cytoplasm	2.82E-13	1.33E-18	0.00025994	1.08E-10
	Nucleus	5.63E-18	2.30E-42	0.0014969	1.91E-22

Table D9. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy ERα data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

		ANAS			DMSO			EP			EPA			EPR			RU486		
		O	N	C	O	N	C	O	N	C	O	N	C	O	N	C	O	N	C
ANAS	O		7.53E-06	0.1211	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24
	N	0.003277		3.39E-08	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24
	C	1	1.48E-05		1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24
DMSO	O	7.91E-22	7.91E-22	7.91E-22		4.77E-15	0.000895 2	2.56E-24	3.93E-24	2.07E-24	2.55E-11	0.8989	1.25E-15	6.27E-22	6.27E-22	6.27E-22	6.85E-23	2.46E-22	2.32E-23
	N	7.91E-22	7.91E-22	7.91E-22	2.07E-12		2.77E-18	2.56E-24	3.76E-24	2.07E-24	7.67E-22	1.16E-07	1.50E-22	6.27E-22	6.27E-22	6.27E-22	1.40E-07	1.85E-05	1.12E-08
	C	7.91E-22	7.91E-22	7.91E-22	0.3894	1.20E-15		1.66E-23	1.77E-22	1.29E-23	5.95E-06	0.1789	3.24E-10	6.27E-22	6.27E-22	6.27E-22	8.43E-24	2.52E-23	4.86E-24
EP	O	7.91E-22	7.91E-22	7.91E-22	1.11E-21	1.11E-21	7.21E-21		1.24E-05	0.2104	1.15E-21	1.46E-23	5.67E-21	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24
	N	7.91E-22	7.91E-22	7.91E-22	1.71E-21	1.64E-21	7.70E-20	0.005412		2.14E-07	1.78E-18	1.10E-21	7.60E-17	1.53E-17	2.30E-18	3.25E-17	1.82E-24	1.82E-24	1.82E-24
	C	7.91E-22	7.91E-22	7.91E-22	8.99E-22	8.99E-22	5.60E-21	1	9.32E-05		1.92E-22	4.09E-24	1.24E-21	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24	1.82E-24
EPA	O	7.89E-22	7.89E-22	7.89E-22	1.11E-08	3.34E-19	0.002589	4.99E-19	7.72E-16	8.35E-20		3.69E-06	0.1684	0.004112	0.01418	0.003554	2.15E-24	2.25E-24	1.98E-24
	N	7.89E-22	7.89E-22	7.89E-22	1	5.06E-05	1	6.34E-21	4.80E-19	1.78E-21	0.001604		7.01E-08	1.42E-09	9.19E-09	8.42E-10	4.18E-20	1.48E-17	5.67E-21
	C	7.89E-22	7.89E-22	7.89E-22	5.42E-13	6.52E-20	1.41E-07	2.47E-18	3.31E-14	5.41E-19	1	3.05E-05		0.04093	0.09183	0.03587	1.89E-24	1.89E-24	1.82E-24
EPR	O	7.91E-22	7.91E-22	7.91E-22	2.73E-19	2.73E-19	2.73E-19	7.91E-22	6.66E-15	7.91E-22	1	6.17E-07	1		0.000719	0.2441	1.82E-24	1.82E-24	1.82E-24
	N	7.91E-22	7.91E-22	7.91E-22	2.73E-19	2.73E-19	2.73E-19	7.91E-22	1.00E-15	7.91E-22	1	4.00E-06	1	0.313		4.71E-05	1.82E-24	1.82E-24	1.82E-24
	C	7.91E-22	7.91E-22	7.91E-22	2.73E-19	2.73E-19	2.73E-19	7.91E-22	1.41E-14	7.91E-22	1	3.66E-07	1	1	0.02048		1.82E-24	1.82E-24	1.82E-24
RU486	O	7.91E-22	7.91E-22	7.91E-22	2.98E-20	6.07E-05	3.67E-21	7.91E-22	7.91E-22	7.91E-22	9.37E-22	1.82E-17	8.24E-22	7.91E-22	7.91E-22	7.91E-22		0.1357	0.2561
	N	7.91E-22	7.91E-22	7.91E-22	1.07E-19	0.008051	1.10E-20	7.91E-22	7.91E-22	7.91E-22	9.78E-22	6.42E-15	8.24E-22	7.91E-22	7.91E-22	7.91E-22	1		0.01537
	C	7.91E-22	7.91E-22	7.91E-22	1.01E-20	4.87E-06	2.11E-21	7.91E-22	7.91E-22	7.91E-22	8.60E-22	2.47E-18	7.89E-22	7.91E-22	7.91E-22	7.91E-22	1	1	

Table D10. The p-values for the Mann Whitney pairwise tests performed on the combined hormone-therapy and immunotherapy ER α data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

		ANAS+NK			DMSO+NK			EP+NK			EPA+NK			EPR+NK			RU486+NK		
		O	N	C	O	N	C	O	N	C	O	N	C	O	N	C	O	N	C
ANAS+NK	O		1.14E-96	8.62E-19	0.001295	1.88E-101	0.000416	8.40E-68	7.07E-243	1.15E-17	8.75E-22	1.11E-135	9.58E-06	1.68E-109	2.82E-242	2.85E-56	9.09E-14	3.01E-66	2.23E-59
	N	4.96E-94		2.81E-171	1.95E-31	6.42E-24	3.44E-74	0.007294	2.89E-73	1.53E-34	2.74E-12	2.11E-20	1.05E-31	2.71E-08	3.35E-81	0.09152	7.98E-119	0.7262	1.23E-191
	C	3.75E-16	1.22E-168		2.79E-19	5.97E-134	0.006508	2.21E-130	2.96E-305	1.34E-61	5.63E-57	2.75E-191	8.38E-28	7.59E-168	5.76E-302	3.65E-106	0.917	2.10E-115	1.43E-20
DMSO+NK	O	0.5633	8.50E-29	1.21E-16		4.32E-53	1.82E-07	5.42E-21	2.35E-114	0.006032	3.21E-06	3.54E-61	0.4063	8.70E-45	1.20E-118	2.93E-19	1.13E-15	5.60E-25	4.65E-44
	N	8.17E-99	2.79E-21	2.60E-131	1.88E-50		3.70E-82	6.77E-31	0.008691	5.17E-64	1.96E-37	0.005604	3.68E-54	3.24E-08	2.51E-05	3.69E-24	2.70E-104	4.08E-17	1.19E-133
	C	0.181	1.49E-71	1	7.93E-05	1.61E-79		7.59E-57	1.90E-167	1.27E-21	6.04E-25	4.20E-104	1.45E-10	1.14E-84	1.26E-168	3.71E-49	0.01179	2.93E-57	3.06E-20
EP+NK	O	3.65E-65	1	9.60E-128	2.36E-18	2.94E-28	3.30E-54		1.75E-86	1.06E-20	5.59E-06	1.75E-29	3.04E-20	9.26E-15	2.98E-93	0.4243	5.25E-95	0.009095	4.57E-161
	N	3.08E-240	1.26E-70	1.29E-302	1.02E-111	1	8.25E-165	7.62E-84		4.31E-159	2.13E-91	1.85E-12	3.39E-125	2.83E-28	0.02549	3.07E-67	1.52E-219	5.33E-49	9.23E-270
	C	5.02E-15	6.67E-32	5.84E-59	1	2.25E-61	5.52E-19	4.62E-18	1.87E-156		0.005647	8.18E-77	0.02944	1.39E-53	1.16E-160	2.21E-18	1.05E-45	1.19E-25	8.12E-107
EPA+NK	O	3.80E-19	1.19E-09	2.45E-54	0.001395	8.52E-35	2.63E-22	0.00243	9.26E-89	1		1.06E-40	9.10E-06	1.56E-24	4.41E-99	2.16E-05	4.52E-44	8.37E-10	1.01E-92
	N	4.81E-133	9.18E-18	1.19E-188	1.54E-58	1	1.83E-101	7.61E-27	8.06E-10	3.56E-74	4.62E-38		7.05E-66	0.0002927	3.42E-18	1.66E-22	2.03E-144	3.02E-14	1.10E-196
	C	0.004168	4.59E-29	3.65E-25	1	1.60E-51	6.32E-08	1.32E-17	1.48E-122	1	0.003959	3.07E-63		7.89E-47	4.23E-132	6.43E-18	6.74E-22	2.55E-24	1.08E-61
EPR+NK	O	7.33E-107	1.18E-05	3.30E-165	3.79E-42	1.41E-05	4.96E-82	4.03E-12	1.23E-25	6.03E-51	6.76E-22	0.1273	3.43E-44		9.42E-37	7.15E-11	8.78E-122	1.60E-05	3.08E-179
	N	1.23E-239	1.46E-78	2.50E-299	5.23E-116	0.01093	5.50E-166	1.30E-90	1	5.05E-158	1.92E-96	1.49E-15	1.84E-129	4.10E-34		1.13E-76	4.10E-217	7.26E-55	2.32E-266
	C	1.24E-53	1	1.59E-103	1.28E-16	1.60E-21	1.61E-46	1	1.34E-64	9.60E-16	0.009385	7.22E-20	2.80E-15	3.11E-08	4.90E-74		1.19E-77	0.08186	2.15E-133
RU486+NK	O	3.95E-11	3.47E-116	1	4.91E-13	1.18E-101	1	2.29E-92	6.61E-217	4.55E-43	1.97E-41	8.85E-142	2.93E-19	3.82E-119	1.78E-214	5.19E-75		3.43E-89	2.99E-16
	N	1.31E-63	1	9.15E-113	2.44E-22	1.77E-14	1.27E-54	1	2.32E-46	5.18E-23	3.64E-07	1.31E-11	1.11E-21	0.006954	3.16E-52	1	1.49E-86		3.88E-143
	C	9.71E-57	5.36E-189	6.23E-18	2.02E-41	5.19E-131	1.33E-17	1.99E-158	4.01E-267	3.53E-104	4.39E-90	4.78E-194	4.68E-59	1.34E-176	1.01E-263	9.35E-131	1.30E-13	1.69E-140	

Table D11. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy compared to the combined hormone-therapy and immunotherapy ER α expression nucleus data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

	ANAS+NK	DMSO+NK	EP+NK	EPA+NK	EPR+NK	RU486+NK	ANAS	DMSO	EP	EPA	EPR	RU486
ANAS+NK		6.42E-24	2.89E-73	2.11E-20	3.35E-81	0.7262	6.56E-26	0	3.14E-25	0.000145	5.42E-157	0
DMSO+NK	4.24E-22		0.008691	0.005604	2.51E-05	4.08E-17	5.35E-10	1.10E-201	1.96E-40	1.23E-19	8.68E-88	4.24E-22
EP+NK	1.91E-71	0.5736		1.85E-12	0.02549	5.33E-49	4.99E-10	0	6.18E-86	5.14E-48	1.15E-112	1.91E-71
EPA+NK	1.39E-18	0.3699	1.22E-10		3.42E-18	3.02E-14	6.51E-16	0	5.77E-48	1.69E-20	4.39E-127	1.39E-18
EPR+NK	2.21E-79	0.001658	1	2.25E-16		7.26E-55	1.02E-07	0	2.07E-86	1.74E-52	1.05E-97	2.21E-79
RU486+NK	1	2.69E-15	3.52E-47	1.99E-12	4.79E-53		7.82E-24	1.703E-310	4.13E-21	0.000224	9.24E-134	1
ANAS	4.33E-24	3.53E-08	3.29E-08	4.30E-14	6.73E-06	5.16E-22		8.75E-50	7.48E-32	4.52E-23	2.26E-09	4.33E-24
DMSO	0	7.23E-200	0	0	0	0	5.78E-48		1.51E-190	5.06E-213	1.13E-160	0
EP	2.07E-23	1.30E-38	4.08E-84	3.81E-46	1.37E-84	2.73E-19	4.93E-30	9.99E-189		1.31E-05	4.72E-119	2.07E-23
EPA	0.009574	8.09E-18	3.39E-46	1.12E-18	1.15E-50	0.01481	2.98E-21	3.34E-211	0.000866		1.79E-112	0.009574
EPR	3.58E-155	5.73E-86	7.61E-111	2.90E-125	6.93E-96	6.10E-132	1.49E-07	7.48E-159	3.11E-117	1.18E-110		3.58E-155
RU486	0	6.42E-24	2.89E-73	2.11E-20	3.35E-81	0.7262	6.56E-26	0	3.14E-25	0.000145	5.42E-157	

Table D12. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy compared to combined hormone-therapy and immunotherapy ER α expression cytoplasm data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

	ANAS+NK	DMSO+NK	EP+NK	EPA+NK	EPR+NK	RU486+NK	ANAS	DMSO	EP	EPA	EPR	RU486
ANAS+NK		0.006508	1.34E-61	8.38E-28	3.65E-106	1.43E-20	1.34E-06	0	6.66E-124	6.42E-59	4.10E-178	0
DMSO+NK	0.4295		1.27E-21	1.45E-10	3.71E-49	3.06E-20	0.00182	1.44E-205	1.41E-83	1.37E-45	1.48E-151	0.4295
EP+NK	8.85E-60	8.37E-20		0.02944	2.21E-18	8.12E-107	0.4168	0	5.48E-186	1.22E-144	1.41E-164	8.85E-60
EPA+NK	5.53E-26	9.59E-09	1		6.43E-18	1.08E-61	0.8051	0	4.71E-146	5.99E-96	4.00E-156	5.53E-26
EPR+NK	2.41E-104	2.45E-47	1.46E-16	4.25E-16		2.15E-133	0.000214	0	1.03E-184	1.54E-154	6.65E-153	2.41E-104
RU486+NK	9.45E-19	2.02E-18	5.36E-105	7.10E-60	1.42E-131		6.13E-15	1.455E-310	5.03E-68	1.65E-16	7.32E-159	9.45E-19
ANAS	8.83E-05	0.1201	1	1	0.01414	4.05E-13		4.25E-49	3.68E-36	5.50E-25	6.65E-43	8.83E-05
DMSO	0	9.51E-204	0	0	0	0.00E+00	2.80E-47		9.26E-148	4.88E-240	3.96E-159	0
EP	4.40E-122	9.31E-82	3.62E-184	3.11E-144	6.77E-183	3.32E-66	2.43E-34	6.11E-146		7.02E-24	1.13E-129	4.40E-122
EPA	4.24E-57	9.02E-44	8.06E-143	3.95E-94	1.02E-152	1.09E-14	3.63E-23	3.22E-238	4.63E-22		5.91E-139	4.24E-57
EPR	2.70E-176	9.79E-150	9.33E-163	2.64E-154	4.39E-151	4.83E-157	4.39E-41	2.61E-157	7.48E-128	3.90E-137		2.70E-176
RU486	0	0.006508	1.34E-61	8.38E-28	3.65E-106	1.43E-20	1.34E-06	0	6.66E-124	6.42E-59	4.10E-178	

Table D13. The MFI values for MUC1 expression

Treatment	Overall (Cell)	Cytoplasm	Nucleus
Media B	0.059778	0.05733	0.073299
dDMSO	0.009678	0.009527	0.010388
EP	0.00864	0.008629	0.008699
ANAS	0.029673	0.028691	0.034401
RU486	0.009015	0.009009	0.008684
EPA	0.008613	0.008604	0.008716
EPR	0.008635	0.008618	0.009032
dDMSO+NK	0.008914347	0.00884887	0.009308086
EP+NK	0.009468437	0.009339088	0.009856825
ANAS+NK	0.009031498	0.00894496	0.009316275
RU486+NK	0.008795786	0.008723785	0.009188384
EPA+NK	0.009363321	0.009273702	0.009632213
EPR+NK	0.009330416	0.009244919	0.00958858

Table D14. The p-values for the normality tests performed on the MUC1data

Treatment	Category	Shapiro-Wilk	Jaque-Bera	Chi ²	Anderson-Darling
dDMSO	Overall	8.56E-08	0.00449	5.27E-07	3.31E-12
	Cytoplasm	2.80E-08	0.005463	2.04E-06	6.80E-14
	Nucleus	8.96E-10	4.67E-68	0.002599	5.91E-10
EP	Overall	1.22E-25	4.83E-84	2.42E-17	7.34E-64
	Cytoplasm	7.65E-26	1.83E-86	6.03E-19	6.02E-65
	Nucleus	6.80E-25	7.19E-247	3.95E-08	1.48E-43
ANAS	Overall	0.8121	0.8599	0.715	0.8928
	Cytoplasm	0.9293	0.9007	0.27332	0.8771
	Nucleus	0.7984	0.8442	0.11146	0.4778
RU486	Overall	0.004514	0.1111	0.30411	0.09886
	Cytoplasm	1.28E-07	3.15E-10	0.016106	6.16E-07
	Nucleus	0.03354	0.05985	0.15825	0.06837
EPA	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	8.12E-32	4.39E-132	6.77E-174	7.17E-152
	Nucleus	<0.0001	<0.001	<0.001	<0.005
EPR	Overall	1.07E-33	1.78E-157	4.51E-163	6.84E-159
	Cytoplasm	1.43E-33	9.78E-151	5.62E-154	1.28E-157
	Nucleus	5.94E-34	2.60E-186	4.32E-189	1.73E-158
dDMSO+NK	Overall	2.28E-54	0	1.25E-215	1.29E-115
	Cytoplasm	1.72E-54	0	1.59E-207	4.45E-114
	Nucleus	2.42E-51	0	5.54E-172	4.61E-170
EP+NK	Overall	1.53E-35	0	5.25E-31	1.22E-95
	Cytoplasm	2.54E-28	4.18E-81	1.74E-17	2.94E-67
	Nucleus	6.44E-46	0	6.55E-81	4.94E-168
ANAS+NK	Overall	1.54E-28	3.88E-147	3.25E-15	7.73E-62
	Cytoplasm	2.68E-22	5.54E-55	1.12E-08	7.60E-38
	Nucleus	<0.0001	<0.001	<0.001	<0.005
RU486+NK	Overall	3.14E-20	8.55E-34	5.70E-11	8.82E-30
	Cytoplasm	1.68E-24	4.96E-58	1.76E-09	4.23E-52
	Nucleus	3.69E-37	0	3.42E-52	1.05E-120
EPA+NK	Overall	<0.0001	<0.001	<0.001	<0.005
	Cytoplasm	<0.0001	<0.001	<0.001	<0.005
	Nucleus	<0.0001	<0.001	<0.001	<0.005
EPR+NK	Overall	3.86E-29	2.63E-115	8.37E-14	3.66E-66
	Cytoplasm	8.01E-31	2.99E-162	1.21E-16	1.21E-73
	Nucleus	2.23E-36	0	2.78E-17	9.06E-87

Table D15. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy MUC1 data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

		ANAS			DMSO			EP			EPA			EPR			RU486		
		O	N	C	O	N	C	O	N	C	O	N	C	O	N	C	O	N	C
ANAS	O		7.22E-06	0.1335	4.28E-16	4.28E-16	4.28E-16	9.82E-21	1.00E-20	9.82E-21	3.31E-20	3.31E-20	3.31E-20	1.84E-20	1.90E-20	1.84E-20	1.32E-19	1.24E-19	1.32E-19
	N	0.003141		1.87E-07	4.28E-16	4.28E-16	4.28E-16	9.82E-21	1.00E-20	9.82E-21	3.31E-20	3.31E-20	3.31E-20	1.84E-20	1.90E-20	1.84E-20	1.32E-19	1.24E-19	1.32E-19
	C	1	8.15E-05		4.28E-16	4.28E-16	4.28E-16	9.82E-21	1.00E-20	9.82E-21	3.31E-20	3.31E-20	3.31E-20	1.84E-20	1.90E-20	1.84E-20	1.32E-19	1.24E-19	1.32E-19
DMSO	O	1.86E-13	1.86E-13	1.86E-13		9.37E-07	0.03255	4.84E-45	3.67E-39	3.47E-46	1.50E-37	2.45E-35	7.61E-38	1.13E-34	2.39E-31	5.47E-36	0.002636	0.0006902	0.003529
	N	1.86E-13	1.86E-13	1.86E-13	0.0004074		6.10E-08	2.96E-53	7.11E-53	2.84E-53	2.43E-48	1.42E-47	1.52E-48	1.59E-47	4.79E-43	2.37E-48	2.05E-38	1.07E-34	8.76E-40
	C	1.86E-13	1.86E-13	1.86E-13	1	2.65E-05		3.30E-34	1.14E-25	1.23E-35	9.58E-34	4.50E-31	4.16E-34	5.16E-31	1.64E-28	4.20E-32	0.8087	0.417	0.9601
EP	O	4.27E-18	4.27E-18	4.27E-18	2.10E-42	1.29E-50	1.43E-31		2.21E-31	0.009524	5.40E-37	0.5208	1.86E-46	1.78E-48	4.32E-09	4.35E-56	1.09E-135	3.21E-129	6.26E-139
	N	4.36E-18	4.36E-18	4.36E-18	1.60E-36	3.09E-50	4.95E-23	9.61E-29		1.56E-41	2.02E-79	7.60E-30	9.34E-83	7.71E-71	7.91E-43	3.54E-76	1.12E-118	2.28E-109	1.39E-123
	C	4.27E-18	4.27E-18	4.27E-18	1.51E-43	1.24E-50	5.33E-33	1	6.81E-39		6.60E-25	0.008427	6.21E-34	3.88E-41	0.0002054	1.95E-49	2.57E-138	1.30E-132	6.55E-141
EPA	O	1.44E-17	1.44E-17	1.44E-17	6.52E-35	1.06E-45	4.17E-31	2.35E-34	8.79E-77	2.87E-22		4.66E-52	0.001407	1.86E-13	5.78E-12	5.23E-21	1.89E-91	2.69E-92	7.03E-92
	N	1.44E-17	1.44E-17	1.44E-17	1.06E-32	6.16E-45	1.96E-28	1	3.31E-27	1	2.03E-49		2.25E-57	9.44E-46	8.82E-13	4.62E-50	1.30E-78	5.53E-82	5.13E-78
	C	1.44E-17	1.44E-17	1.44E-17	3.31E-35	6.59E-46	1.81E-31	8.09E-44	4.06E-80	2.70E-31	0.6122	9.80E-55		3.76E-08	9.56E-20	5.02E-15	2.99E-93	1.15E-93	1.46E-93
EPR	O	7.99E-18	7.99E-18	7.99E-18	4.92E-32	6.92E-45	2.24E-28	7.74E-46	3.35E-68	1.69E-38	8.09E-11	4.11E-43	1.63E-05		5.80E-31	0.008481	4.36E-79	1.07E-81	3.14E-78
	N	8.26E-18	8.26E-18	8.26E-18	1.04E-28	2.08E-40	7.14E-26	1.88E-06	3.44E-40	0.08933	2.51E-09	3.84E-10	4.16E-17	2.52E-28		1.95E-39	1.10E-58	6.19E-63	2.58E-57
	C	7.99E-18	7.99E-18	7.99E-18	2.38E-33	1.03E-45	1.83E-29	1.89E-53	1.54E-73	8.47E-47	2.27E-18	2.01E-47	2.19E-12	1	8.50E-37		1.14E-85	1.12E-87	6.72E-85
RU486	O	5.75E-17	5.75E-17	5.75E-17	1	8.89E-36	1	4.76E-133	4.87E-116	1.12E-135	8.24E-89	5.67E-76	1.30E-90	1.90E-76	4.80E-56	4.97E-83		0.4374	0.5749
	N	5.38E-17	5.38E-17	5.38E-17	0.3003	4.63E-32	1	1.40E-126	9.92E-107	5.64E-130	1.17E-89	2.41E-79	5.02E-91	4.63E-79	2.69E-60	4.87E-85	1		0.232
	C	5.75E-17	5.75E-17	5.75E-17	1	3.81E-37	1	2.72E-136	6.03E-121	2.85E-138	3.06E-89	2.23E-75	6.37E-91	1.37E-75	1.12E-54	2.92E-82	1	1	

Table D16. The p-values for the Mann Whitney pairwise tests performed on the combined hormone-therapy and immunotherapy MUC1 data ($p < 0.001$) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

		ANAS+NK			DMSO+NK			EP+NK			EPA+NK			EPR+NK			RU486+NK		
		O	N	C	O	N	C	O	N	C	O	N	C	O	N	C	O	N	C
ANAS+NK	O		1.45E-110	7.10E-19	6.73E-26	1.04E-166	3.30E-09	6.53E-226	0	5.55E-134	2.16E-57	1.44E-193	3.68E-25	6.00E-167	0	5.55E-97	2.56E-123	1.24E-17	4.84E-219
	N	6.32E-108		1.63E-198	3.25E-06	1.23E-41	1.39E-17	7.25E-32	2.08E-181	0.0008114	0.0001454	3.77E-29	3.05E-21	2.12E-06	5.79E-95	0.01593	0	3.15E-24	0
	C	3.09E-16	7.08E-196		9.25E-57	9.27E-222	3.92E-29	0	0	8.46E-222	1.67E-118	7.94E-282	3.35E-71	3.16E-271	0	1.20E-186	1.10E-56	1.08E-55	7.83E-132
DMSO+NK	O	2.93E-23	0.001416	4.02E-54		1.05E-40	0.001371	5.51E-31	1.05E-118	1.82E-09	0.01198	3.90E-39	0.1395	3.09E-11	1.11E-66	0.02523	4.30E-127	0.002785	4.00E-183
	N	4.53E-164	5.33E-39	4.03E-219	4.57E-38		5.51E-56	5.96E-09	1.99E-14	5.42E-31	3.25E-43	0.001873	7.80E-69	3.99E-32	0.8213	1.62E-59	2.29E-287	9.06E-76	0
	C	1.44E-06	6.03E-15	1.71E-26	0.5962	2.40E-53		6.64E-49	1.35E-142	1.35E-21	1.25E-10	4.69E-60	0.008391	2.60E-24	1.42E-89	1.31E-09	9.76E-86	0.5056	6.84E-136
EP+NK	O	2.84E-223	3.15E-29	0	2.40E-28	2.59E-06	2.89E-46		2.33E-75	2.77E-16	5.36E-38	0.173	7.82E-74	3.26E-16	1.89E-18	2.11E-52	0	8.57E-85	0
	N	0	9.03E-179	0	4.58E-116	8.67E-12	5.85E-140	1.01E-72		1.32E-143	6.71E-163	2.42E-46	2.09E-221	5.91E-164	1.04E-27	5.63E-244	0	3.89E-232	0
	C	2.41E-131	0.353	3.68E-219	7.93E-07	2.36E-28	5.88E-19	1.21E-13	5.73E-141		5.59E-09	5.26E-18	2.30E-29	0.5267	5.69E-65	1.68E-10	0	1.28E-36	0
EPA+NK	O	9.40E-55	0.06325	7.27E-116	1	1.41E-40	5.42E-08	2.33E-35	2.92E-160	2.43E-06		2.07E-40	2.57E-07	3.77E-13	8.51E-92	0.1622	4.39E-242	1.40E-09	0
	N	6.25E-191	1.64E-26	3.46E-279	1.70E-36	0.8148	2.04E-57	1	1.05E-43	2.29E-15	9.01E-38		1.16E-73	1.67E-15	1.37E-08	4.09E-43	0	6.68E-74	0
	C	1.60E-22	1.33E-18	1.46E-68	1	3.39E-66	1	3.40E-71	9.07E-219	1.00E-26	0.0001117	5.07E-71		4.52E-39	4.22E-144	3.44E-13	1.75E-187	0.1874	2.22E-283
EPR+NK	O	2.61E-164	0.0009201	1.38E-268	1.35E-08	1.73E-29	1.13E-21	1.42E-13	2.57E-161	1	1.64E-10	7.24E-13	1.96E-36		9.52E-74	1.55E-15	0	3.03E-48	0
	N	0	2.52E-92	0	4.82E-64	1	6.17E-87	8.24E-16	4.53E-25	2.48E-62	3.70E-89	5.95E-06	1.83E-141	4.14E-71		3.42E-140	0	1.21E-156	0
	C	2.42E-94	1	5.21E-184	1	7.06E-57	5.69E-07	9.17E-50	2.45E-241	7.31E-08	1	1.78E-40	1.50E-10	6.74E-13	1.49E-137		0	1.33E-18	0
RU486+NK	O	1.11E-120	0	4.78E-54	1.87E-124	9.94E-285	4.24E-83	0	0	0	1.91E-239	0	7.61E-185	0	0	0		3.35E-161	6.61E-20
	N	5.40E-15	1.37E-21	4.68E-53	1	3.94E-73	1	3.73E-82	1.69E-229	5.59E-34	6.07E-07	2.91E-71	1	1.32E-45	5.28E-154	5.80E-16	1.46E-158		4.68E-238
	C	2.11E-216	0	3.41E-129	1.74E-180	0	2.98E-133	0	0	0	0	0	9.67E-281	0	0	0	2.88E-17	2.03E-235	

Table D17. The p-values for the Mann Whitney pairwise tests performed on the hormone-therapy compared to combined hormone-therapy and immunotherapy MUC1 expression nucleus data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

	ANAS+NK	DMSO+NK	EP+NK	EPA+NK	EPR+NK	RU486+NK	ANAS	DMSO	EP	EPA	EPA	RU486
ANAS+NK		1.23E-41	2.08E-181	3.77E-29	5.79E-95	3.15E-24	3.87E-21	6.00E-21	0	1.69E-207	1.78E-197	3.91E-21
DMSO+NK	9.56E-40		1.99E-14	0.001873	0.8213	9.06E-76	5.53E-21	7.73E-08	4.84E-269	5.64E-166	5.08E-168	9.30E-45
EP+NK	1.62E-179	1.56E-12		2.42E-46	1.04E-27	3.89E-232	3.92E-21	0.001022	0	9.37E-252	9.10E-267	3.50E-108
EPA+NK	2.94E-27	0.1461	1.89E-44		1.37E-08	6.68E-74	3.97E-21	2.76E-11	0	1.49E-216	2.31E-218	2.50E-38
EPR+NK	4.51E-93	1	8.11E-26	1.07E-06		1.21E-156	3.68E-21	2.40E-08	0	1.51E-245	8.15E-252	3.68E-81
RU486+NK	2.46E-22	7.07E-74	3.03E-230	5.21E-72	9.46E-155		4.94E-21	1.56E-27	5.92E-201	1.14E-136	2.61E-130	0.002622
ANAS	3.02E-19	4.31E-19	3.06E-19	3.10E-19	2.87E-19	3.85E-19		4.28E-16	1.00E-20	3.31E-20	1.90E-20	1.24E-19
DMSO	4.68E-19	6.03E-06	0.07974	2.15E-09	1.87E-06	1.21E-25	3.34E-14		7.11E-53	1.42E-47	4.79E-43	1.07E-34
EP	0	3.78E-267	0	0	0	4.62E-199	7.81E-19	5.54E-51		7.60E-30	7.91E-43	2.28E-109
EPA	1.31E-205	4.40E-164	7.31E-250	1.16E-214	1.18E-243	8.86E-135	2.58E-18	1.11E-45	5.93E-28		8.82E-13	5.53E-82
EPR	1.39E-195	3.96E-166	7.10E-265	1.80E-216	6.36E-250	2.04E-128	1.48E-18	3.73E-41	6.17E-41	6.88E-11		6.19E-63
RU486	3.05E-19	7.26E-43	2.73E-106	1.95E-36	2.87E-79	0.2045	9.64E-18	8.31E-33	1.78E-107	4.31E-80	4.83E-61	

Table D18. The p-values for the Mann-Whitney pairwise comparison tests performed on the hormone-therapy compared to hormone-therapy and immunotherapy MUC1 expression cytoplasm data (p<0.001) (Bonferroni corrected \ uncorrected; Bonferroni corrected values used)

	ANAS+NK	DMSO+NK	EP+NK	EPA+NK	EPR+NK	RU486+NK	ANAS	DMSO	EP	EPA	EPA	RU486
ANAS+NK		3.92E-29	8.46E-222	3.35E-71	1.20E-186	7.83E-132	3.87E-21	2.75E-05	3.07E-199	2.38E-129	1.58E-141	2.84E-10
DMSO+NK	3.06E-27		1.35E-21	0.008391	1.31E-09	6.84E-136	5.53E-21	0.02136	3.16E-165	8.11E-117	2.54E-130	0.1348
EP+NK	6.60E-220	1.05E-19		2.30E-29	1.68E-10	0	3.92E-21	0.4851	0	3.97E-225	1.81E-255	3.02E-35
EPA+NK	2.61E-69	0.6545	1.79E-27		3.44E-13	2.22E-283	3.97E-21	0.3671	2.13E-314	1.35E-191	1.23E-212	0.001454
EPR+NK	9.34E-185	1.02E-07	1.31E-08	2.68E-11		0	3.68E-21	0.8778	0	2.34E-221	2.80E-249	8.86E-29
RU486+NK	6.11E-130	5.34E-134	0	1.73E-281	0		4.94E-21	6.82E-20	1.06E-17	8.75E-31	4.07E-41	1.88E-87
ANAS	3.02E-19	4.31E-19	3.06E-19	3.10E-19	2.87E-19	3.85E-19		4.28E-16	9.82E-21	3.31E-20	1.84E-20	1.32E-19
DMSO	0.002146	1	1	1	1	5.32E-18	3.34E-14		1.23E-35	4.16E-34	4.20E-32	0.9601
EP	2.40E-197	2.46E-163	0	1.662E-312	0	8.28E-16	7.66E-19	9.57E-34		6.21E-34	1.95E-49	6.55E-141
EPA	1.86E-127	6.33E-115	3.09E-223	1.06E-189	1.83E-219	6.83E-29	2.58E-18	3.25E-32	4.84E-32		5.02E-15	1.46E-93
EPA	1.23E-139	1.98E-128	1.41E-253	9.58E-211	2.18E-247	3.17E-39	1.43E-18	3.28E-30	1.52E-47	3.92E-13		6.72E-85
RU486	2.22E-08	1	2.35E-33	0.1134	6.91E-27	1.46E-85	1.03E-17	1	5.11E-139	1.14E-91	5.24E-83	

Table D19. Spearman's correlation values (>0.8 = very strong correlation)

 >0.8 Strong correlation

Treatment	PR and MUC1			ERα and MUC1		
	Cell	Nuclei	Cytoplasm	Cell	Nuclei	Cytoplasm
Media B	0.26358	0.26534	0.24547	0.80345	0.74945	0.81993
dDMSO	0.65613	0.63834	0.63794	0.045766	-0.32812	0.31761
EP	0.62598	0.73607	0.54462	0.72285	0.64565	0.71779
ANAS	0.82469	0.68988	0.83849	-0.11666	-0.09598	0.08725
RU486	0.90168	0.87731	0.91569	0.85537	0.91687	0.79828
EPA	0.77809	0.56283	0.79516	0.52863	0.50962	0.52706
EPR	0.25918	0.86674	-0.2603	-0.16552	-0.23797	-0.10123
dDMSO+NK	0.739	0.62803	0.7504	0.7994	0.82531	0.78399
EP+NK	0.87304	0.93143	0.81242	0.90408	0.92779	0.88392
ANAS+NK	0.69715	0.8247	0.64088	0.91365	0.89503	0.91762
RU486+NK	0.9144	0.97233	0.91122	0.91836	0.90047	0.93153
EPA+NK	0.83769	0.85363	0.84121	0.83239	0.81633	0.82742
EPR+NK	0.78676	0.86093	0.77719	0.93116	0.93997	0.92311

Table D20. The Ki67⁺ values

Treatment	% Ki67⁺
Media B	4.3
dDMSO	2.0
EP	3.0
ANAS	4.3
RU486	2.5
EPA	2.6
EPR	5.0
ANAS+NK	0.9
RU486+NK	2.1
EPA+NK	2.0
EPR+NK	3.7

Table D21. The p-values for the normality tests performed on the Ki67 data ($p < 0.05$)

Treatment	Shapiro-Wilk	Jaque-Bera	Anderson-Darling
dDMSO	0.656	0.8405	0.5033
EP	0.9178	0.9041	0.698
ANAS	0.31	0.786	0.285
RU486	0.6704	0.8086	0.5588
EPA	0.5329	0.7781	0.4295
EPR	0.7682	0.821	0.6545
dDMSO+NK	0.04432	0.6455	0.04629
EP+NK	0.4919	0.7872	0.3322
ANAS+NK	0.3624	0.6488	0.3462
RU486+NK	0.8232	0.8355	0.784
EPA+NK	0.1057	0.6242	0.1165
EPR+NK	0.1243	0.6166	0.08019

Table D22. The values for the Tukey's pairwise tests performed on the Ki67 data (significance set at $p < 0.05$) (Q \ p-value)

	EPA	EPR	EP	ANAS	RU486	DMSO	Media B	EPA+NK	EPR+NK	EP+NK	ANAS+NK	RU486+NK
EPA		1	1	1	1	0.9894	1	0.9903	1	0.9296	0.5439	0.9935
EPR	0.6138		0.9999	1	1	0.9001	1	0.9048	1	0.7208	0.2759	0.9235
EP	0.5088	1.123		1	1	0.9996	0.9996	0.9996	1	0.9903	0.7798	0.9998
ANAS	0.4019	1.016	0.1069		1	0.9991	0.9998	0.9992	1	0.984	0.7343	0.9996
RU486	0.3713	0.2425	0.8801	0.7732		0.9519	1	0.9547	1	0.8209	0.3718	0.9657
DMSO	1.815	2.429	1.306	1.413	2.186		0.8465	1	0.9296	1	0.9985	1
Media B	0.7898	0.176	1.299	1.192	0.4185	2.605		0.8526	1	0.6387	0.2171	0.8773
EPA+NK	1.797	2.411	1.288	1.395	2.169	0.1788	2.587		0.9333	1	0.9983	1
EPR+NK	0.4905	0.1234	0.9993	0.8924	0.1192	2.306	0.2993	2.288		0.7741	0.3226	0.9479
EP+NK	2.305	2.919	1.797	1.904	2.677	0.4903	3.095	0.5081	2.796		1	1
ANAS+NK	3.291	3.905	2.782	2.889	3.662	1.476	4.081	1.493	3.781	0.9853		0.9973
RU486+NK	1.72	2.333	1.211	1.318	2.091	0.09567	2.509	0.07779	2.21	0.5859	1.571	

Table D23. The mean percentage values for each quadrant (Q1: Annexin⁺ PI⁻, Q2: Annexin⁺ PI⁺, Q3: Annexin⁻ PI⁺, Q4: Annexin⁻ PI⁻) in all treatment groups

Treatment	Q1	Q2	Q3	Q4
Media B	4.31	15.57	8.25	71.87
dDMSO	12.45	14.70	8.27	64.63
CPT	2.66	11.69	3.87	81.77
EP	5.70	11.83	8.21	74.27
ANAS	4.84	11.52	5.02	78.60
RU486	3.00	11.75	9.78	75.43
EPA	8.73	13.23	5.33	72.73
EPR	2.16	9.55	8.27	80.00
Media B+NK	4.28	26.00	6.82	62.93
dDMSO+NK	3.98	10.20	12.55	73.27
EP+NK	5.80	7.93	3.94	82.30
ANAS+NK	4.69	9.05	6.60	79.67
RU486+NK	2.87	10.07	9.07	78.00
EPA+NK	4.34	7.57	4.82	83.30
EPR+NK	2.12	10.29	6.79	80.80

Table D24. The p-values for the normality tests performed on the quadrant data (Q1: Annexin⁺ PI⁻, Q2: Annexin⁺ PI⁺, Q3: Annexin⁻ PI⁺, Q4: Annexin⁻ PI⁻) ($p < 0.05$)

Treatment	Quadrant	Shapiro-Wilk	Jaque-Bera
dDMSO	Q1	0.502	0.8165
	Q2	0.7941	0.8579
	Q3	0.002779	0.7667
	Q4	0.1975	0.7757
EP	Q1	0.4082	0.8018
	Q2	0.9525	0.8682
	Q3	0.1438	0.7716
	Q4	0.03773	0.7671
ANAS	Q1	0.4108	0.8022
	Q2	0.7127	0.8484
	Q3	0.6458	0.839
	Q4	0.6536	0.8402
RU486	Q1	0.4514	0.8085
	Q2	0.6962	0.8462
	Q3	0.1882	0.7749
	Q4	0.3493	0.7933
EPA	Q1	0.2788	0.7842
	Q2	0.2273	0.7785
	Q3	0.3244	0.7899
	Q4	0.8114	0.8596
EPR	Q1	0.9286	0.8675
	Q2	0.1701	0.7734
	Q3	0.1716	0.7736
	Q4	0.7875	0.8572
dDMSO+NK	Q1	0.2136	0.7772
	Q2	0.6801	0.844
	Q3	0.8701	0.8644
	Q4	0.1239	0.7703
EP+NK	Q1	0.376	0.797
	Q2	0.5426	0.823
	Q3	0.6084	0.8334
	Q4	0.154	0.7722
ANAS+NK	Q1	0.9531	0.8682
	Q2	0.1465	0.7717
	Q3	0.8802	0.865
	Q4	0.9277	0.8674
RU486+NK	Q1	0.9082	0.8666
	Q2	0.1321	0.7708
	Q3	0.5788	0.8288
	Q4	0.8632	0.8639
EPA+NK	Q1	0.6079	0.8333
	Q2	0.3399	0.792
	Q3	0.4913	0.8148
	Q4	0.3517	0.7936
EPR+NK	Q1	0.3725	0.7965
	Q2	0.3518	0.7936
	Q3	0.251	0.781
	Q4	0.9186	0.867

Table D25. The p-values for the Kruskal Wallis tests performed on the Q1 data (Annexin⁺ PI⁻) (p<0.05)

	EPA	EPR	EP	ANAS	RU486	DMSO	PRF	CPT	EPA+NK	EPR+NK	EP+NK	ANAS+NK	RU486+NK
EPA													
EPR													
EP	0.8273	0.04953											
ANAS	0.8273												
RU486		0.2752											
DMSO	0.5127	0.04953	0.1266	0.04953	0.04953								
PRF	0.8273	0.1266	0.2752	0.8273	0.2752	0.04953							
CPT						0.04953	0.2752						
EPA+NK	0.5127					0.1266	0.8273						
EPR+NK		0.8273				0.04953	0.2752						
EP+NK			0.8273			0.1266	0.8273						
ANAS+NK				0.8273		0.04953	0.8273		0.8273				
RU486+NK					0.8273	0.04953	0.2752			0.5127			

Table D26. The p-values for the Kruskal Wallis tests performed on the Q2 data (Annexin⁺ PI⁺) (p<0.05)

	EPA	EPR	EP	ANAS	RU486	DMSO	PRF	CPT	EPA+NK	EPR+NK	EP+NK	ANAS+NK	RU486+NK
EPA													
EPR													
EP	0.8273	0.5127											
ANAS	0.8273												
RU486		0.5127											
DMSO	0.5127	0.1266	0.5127	0.5127	0.5127								
PRF	0.5127	0.04953	0.2752	0.1266	0.2612	0.8273							
CPT						0.5127	0.5127						
EPA+NK	0.1266					0.04953	0.04953						
EPR+NK		0.5127				0.5127	0.2752						
EP+NK			0.2752			0.1266	0.04953						
ANAS+NK				0.5127		0.1266	0.04953		0.2752				
RU486+NK					0.5127	0.2752	0.04953			0.5127			

Table D27. The p-values for the Kruskal Wallis tests performed on the Q3 (Annexin⁻ PI⁺) data (p<0.05)

	EPA	EPR	EP	ANAS	RU486	DMSO	PRF	CPT	EPA+NK	EPR+NK	EP+NK	ANAS+NK	RU486+NK
EPA													
EPR													
EP	0.1266	0.8273											
ANAS	0.8273												
RU486		0.8273											
DMSO	0.8273	0.5127	0.5127	0.8273	0.8273								
PRF	0.2752	0.5127	0.8273	0.2752	0.5127	0.5127							
CPT						0.8273	0.04953						
EPA+NK	0.8273					1	0.04953						
EPR+NK		0.5127				0.5127	0.2752						
EP+NK			0.1266			0.8273	0.04953						
ANAS+NK				0.5127		0.8273	0.5127		0.5127				
RU486+NK					0.8273	1	0.8273			0.8273			

Table D28. The p-values for the Kruskal Wallis tests performed on the Q4 (Annexin⁻ PI) data (p<0.05)

	EPA	EPR	EP	ANAS	RU486	DMSO	PRF	CPT	EPA+NK	EPR+NK	EP+NK	ANAS+NK	RU486+NK
EPA													
EPR													
EP	0.8273	0.2752											
ANAS	0.5127												
RU486		0.2752											
DMSO	0.2752	0.04953	0.2752	0.04953	0.04953								
PRF	0.8273	0.1266	0.5127	0.2752	0.2752	0.1266							
CPT						0.2752	0.5127						
EPA+NK	0.1266					0.04953	0.04953						
EPR+NK		0.8273				0.04953	0.2752						
EP+NK			0.2752			0.04953	0.2752						
ANAS+NK				0.8273		0.04953	0.1266		0.5127				
RU486+NK					0.5127	0.04953	0.2752			0.5127			

Table D29. The p-values for the Kruskal Wallis tests performed on the total apoptosis (Q1+Q2) data (Q1: Annexin⁺ PI⁻ , Q2: Annexin⁺ PI⁺) (p<0.05)

	EPA	EPR	EP	ANAS	RU486	DMSO	PRF	CPT	EPA+NK	EPR+NK	EP+NK	ANAS+NK	RU486+NK
EPA													
EPR													
EP	0.5172	0.2752											
ANAS	0.2752												
RU486		0.2752											
DMSO	0.5127	0.04953	0.04953	0.04953	0.04953								
PRF	0.8273	0.04953	0.8273	0.5127	0.2752	0.04953							
CPT						0.1266	0.5127						
EPA+NK	0.1266					0.04953	0.1266						
EPR+NK		0.5127				0.04953	0.1266						
EP+NK			0.3758			0.04953	0.2752						
ANAS+NK				0.5127		0.04953	0.04953		0.5127				
RU486+NK					0.5127	0.04953	0.04953			0.8273			